Enz-52(D2)(C)(D1) PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Engelhardt Examiner: K. Salmon

Serial No.: 10/713,183 Group Art Unit: 1634

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For: *In vitro* Process for Producing Multiple Nucleic Acid Copies

Confirmation No. 5179

AMENDMENT UNDER 37 CFR §1.116

Commissioner of Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

This is in response to the Office Action dated December 24, 2009.

CLAIM AMENDMENTS

Claims 1-111 (Canceled).

112. (Currently Amended) An *in vitro* process for producing more than one copy of a DNA molecule of interest, said process comprising the steps of:
(a) providing a nucleic acid sample containing said DNA molecule of interest;
(b) contacting said sample with a mixture comprising: (i) nucleic acid precursors; (ii) one or more specific polynucleotide primers comprising at least one ribonucleic acid segment, each of which primer comprises a sequence complementary to a distinct sequence of said DNA molecule of interest; (iii) an effective amount of a nucleic acid producing catalyst; and (iv) RNase H;
(c) carrying out nucleic acid synthesis to extend a primer bound to said DNA molecule of interest and produce a polynucleotide comprising an RNNDNA hybrid, thereby generating a substrate for RNase H; and
(d) digesting said substrate with RNase H to remove said ribonucleic acid segment of said extended primer, wherein said removal allows another primer binding event to occur with said one or more specific polynucleotide primers comprising at least one ribonucleic acid segment complementary to said DNA molecule of interest, thereby producing multiple copies of said nucleic acids of interest by means of said steps (c) and (d).

113. (Previously Presented) The process of claim 112 wherein said primers (ii) comprise modified nucleotides. unmodified nucleotides or a combination thereof.

114. (Previously Presented) The process of claim 112, wherein said primers (ii) comprise sequences noncomplementary to said distinct sequence of said DNA molecule of interest.

115. (Previously Presented) The process of claim 114, wherein said primers (ii) comprise from about 1 to 200 non complementary nucleotides or nucleotide analogs.

116. (Previously Presented) The process of claim 112, wherein said primers (ii) further comprise deoxyribonucleotides.

117. (Previously Presented) The process of claim 112, wherein said nucleic acid producing catalysts (iii) comprise DNA polymerase, reverse transcriptase or a combination thereof.

118. (Previously Presented) The process of claim 117, wherein said DNA polymerase comprises E. coli DNA polymerase I, Klenow polymerase, polymerases derived from thermophilic bacteria or a combination thereof.

119. (Previously Presented) The process of claim 118, wherein said polymerases derived from thermophilic bacteria comprise Taq DNA polymerase.

120. (Previously Presented) The process of claim 112, wherein said mixture recited in step (b) comprises labeled nucleic acid precursors, one or more specific labeled polynucleotide primers, or a combination of both.

Claims 121-122 (Canceled)

123. (Currently Amended) A process for multiply initiating polynucleotide or oligonucleotide synthesis of a DNA molecule of interest comprising:
(a) providing a sample containing said DNA molecule of interest;
(b) contacting said sample with a mixture comprising: (i) nucleic acid precursors; (ii) one or more specific copolymer primers comprising at least one DNA segment and at least one RNA segment, each of which primer comprises a sequence complementary to a distinct sequence of said DNA molecule of interest; (iii) an effective amount of a nucleic acid producing catalyst; and (iv) RNase H;

(c) producing at least one copy of said DNA molecule of interest by using said nucleic acid producing catalyst (iii) and said DNA molecules of interest as templates to extend said copolymer primer; and
(d) removing said RNA segment of said extended copolymer primer from said template by digesting with RNase H to bind another copy of said copolymer primer to said template and initiate synthesis,
thereby multiply initiatingso that multiple initiation of polynucleotide or oligonucleotide synthesis occurs.

124. (Previously Presented) The process of claim 123, wherein said primers comprise modified nucleotides, unmodified nucleotides or a combination thereof.

125. (Previously Presented) The process of claim 123, wherein said primers further comprise sequences that are noncomplementary to said DNA molecule of interest.

126. (Previously Presented) The process of claim 125, wherein said primers comprise from about 1 to 200 noncomplementary nucleotides or nucleotide analogs.

127. (Previously Presented) The process of claim 123, wherein the nucleic acid producing catalyst (iii) comprises DNA polymerase, reverse transcriptase or a combination thereof.

128. (Previously Presented) The process of claim 127, wherein sald DNA polymerase comprises E. coli DNA polymerase I, Klenow polymerase, polymerases derived from thermophilic bacteria or a combination thereof.

129. (Previously Presented) The process of claim 128, wherein said polymerases derived from thermophilic bacteria comprise Taq DNA polymerase.

130. (Previously Presented) The process of claim 123, wherein said mixture recited in step (b) comprises labeled nucleic acid precursors, one or more specific labeled

polynucleotide primers or a combination of both.

Claims 131-132 (Canceled).

133. (Currently Amended) An *in vitro* process for producing more than one complementary copy of an RNA molecule of interest, said process comprising the steps of:
(a) providing a nucleic acid sample containing said RNA molecule of interest;
(b) contacting said sample containing said RNA with a mixture comprising: (i) nucleic acid precursors; (ii) one or more polynucleotide primers wherein said primers comprise (A) at least one ribonucleic acid segment and (B) a sequence complementary to a distinct sequence in said RNA molecule of interest; (iii) an effective amount of a nucleic acid producing catalyst; and (iv) RNase H;
(c) producing a first DNA copy from said RNA molecule of interest, by binding said polynucleotide primer with said RNA molecule of interest as a template;
(d) using said first DNA copy as a template to produce a double-stranded nucleic acid comprising a second copy complementary to said DNA copy produced in step (c); and
(e) removing said ribonucleic acid segment of said extended primers with RNase H from said first DNA copy of the double-stranded ~~copy~~ nucleic acid produced in step (d) to generate a primer binding site on said second copy of (~~c~~d) to render said primer binding site available for subsequent primer binding and extension events ~~and producing~~to produce more than one copy of said RNA molecule of interest.

134. (Previously Presented) The process of claim 133, wherein said primers (ii) comprise modified nucleotides, unmodified nucleotides or a combination thereof.

135. (Previously Presented) The process of claim 133, wherein said primers (ii) further comprise sequences noncomplementary to said distinct sequence of said RNA of interest.

136. (Previously Presented) The process of claim 135, wherein said primers (ii) further

comprise from about 1 to 200 noncomplementary nucleotides or nucleotide analogs.

137. (Previously Presented) The process of claim 133, wherein said primers (ii) further comprise deoxyribonucleotides.

138. (Previously. Presented) The process of claim 133, wherein said nucleic acid producing catalysts (iii) comprise DNA polymerase, reverse transcriptase or a combination thereof.

139. (Previously Presented) The process of claim 133, wherein said DNA polymerase comprises E. coli DNA polymerase I, Klenow polymerase, polymerases derived from thermophilic bacteria, or a combination thereof.

140. (Previously Presented) The process of claim 133, wherein said polymerases derived from thermophilic bacteria comprise Taq DNA polymerase.

Claims 141-142 (Canceled).

143. (Currently Amended) An isostatic in vitro process for producing more than one copy of a DNA molecule, said process comprising the steps of:
(a) providing a nucleic acid sample containing or suspected of containing said DNA molecule;
(b) contacting said sample with a mixture comprising: (i) nucleic acid precursors, (ii) specific polynucleotide primers comprising at least one ribonucleic acid segment, each of which primer is substantially complementary to a distinct sequence of said DNA molecule, (iii) an effective amount of a DNA polymerase; and (iv) an effective amount of RNase H;
(c) allowing said mixture to react under isostatic conditions of temperature, buffer and ionic strength, thereby (i) producing at least one copy of said DNA molecule by extension of said primer; and ~~(d)~~(ii) removing ribonucleotides from said ribonucleic acid segment using said RNase H, to regenerate a primer binding site on said DNA

molecule, to render said primer binding site available for another primer binding event to take place at said regenerated primer binding side and thereby producingto produce more than one copy of said DNA molecule under isostatic conditions.

144. (Previously Presented) The method of claim 143, wherein said primers are DNA/RNA copolymers which comprise said RNA segment, and further comprise a DNA segment.

145. (Previously Presented) The method of claim 143, wherein said primers further comprise sequences which are non-complementary to said DNA molecule.

146. (Currently Amended) An isostatic in vitro process for producing more than one copy of a DNA molecule, said process comprising the steps of:
(a) providing a nucleic acid sample containing or suspected of containing said DNA molecule;
(b) contacting said sample with a mixture comprising: (i) nucleic acid precursors, (ii) specific polynucleotide primers comprising at least one ribonucleic acid segment, each of which primer is substantially complementary to a distinct sequence of said DNA molecule, and (iii) an effective amount of a reverse transcriptase having RNase H activity;
(c) allowing said mixture to react under isostatic conditions of temperature, buffer and ionic strength, thereby producing at least one copy of said DNA molecule by extension of said primer; and
(d) removing ribonucleotides from said ribonucleic acid segment using said reverse transcriptase, to regenerate a primer binding site on said DNA molecule, to render said primer binding site available for another primer binding event and thereby producingto produce more than one copy of said DNA molecule under isostatic conditions.

147. (Previously Presented) The method of claim 146, wherein said primers are DNA/RNA copolymers which comprise said RNA segment, and further comprise a DNA segment.

148. (Previously Presented) The method of claim 146. wherein said primers further comprise sequences which are non-complementary to said DNA molecule.

149. (Previously Presented) The process of claim 143, wherein said mixture recited in step (b) comprises labeled nucleic acid precursors, one or more specific labeled polynucleotide primers or a combination of both.

150. (Previously Presented) The process of claim 149, wherein said polynucleotide primers comprise from about 1 to 200 noncomplementary nucleotides or nucleotide analogs.

REMARKS

Applicants would first like to thank Examiner Katherine Salmon for her time and helpful suggestions during the telephonic interview on June 16, 2010 with Applicants' representative, Cheryl H. Agris. The substance of the interview is provided below.

As discussed during the interview and as will be discussed in further detail below, claims 112, 123, 133, 143 and 146 have been amended to more distinctly claim the subject matter of the invention. Applicants reserve the right to file continuation and/or divisional applications containing claims encompassing the canceled subject matter. As will be discussed in further detail below, the amended claims contain no new matter and are supported by the specification.

1. SUBSTANCE OF INTERVIEW

A. Brief Description of any Exhibit Shown or any Demonstration Conducted

No exhibit was shown and no demonstration was conducted.

B. Identification of Claims Discussed

Claims 112, 123, 133, 143 and 146 were discussed.

C. Identification of Specific Prior Art Discussed

As will be set forth in further detail below, Scheele et al., US Patent No. 5,162,209 (hereinafter "Scheele"), Schuster et al., US Patent No. 5,169,766 (hereinafter "Schuster") and Vary et al., US Patent No. 4,851,331 (hereinafter "Vary").

D. Identification of Principal Proposed Amendments of a Substantive Nature Discussed

Amendments to claims 112, 123, 133, 143 and 146 were discussed.

E. Identification of General Thrust of Principal Arguments presented to the examiner

The claim amendments overcome the prior art rejections.

F. A General Indication of Any other Pertinent Matters Discussed

The Examiner requested clarification of the term "isostatic".

G. General Results or Outcome of the Interview

Applicants will present arguments showing that the amended claims are not anticipated by or obvious over the cited references and will clarify the meaning of isostatic.

2. The Rejections Under 35 USC §102

Claims 112-120, 123-130, 143-145, 149-150 are rejected under 35 U.S.C. 102(b) as being anticipated by Scheele. There are three independent claims mentioned: claims 112, 123 and 143. Each of the rejections of the independent claims is discussed in detail below.

2.1 Claims 112-120

The Office Action on pages 11-14 specifically states:

> (B) The reply asserts that there is an important distinction between the instant invention and the method of Scheele, which can be seen in Scheele's figure 5 (p. 21 1st full paragraph of arguments). The reply asserts that in Figure 5, RNase H digestion is used to remove an RNA primer, but the newly exposed single stranded region is not used for binding of another RNA primer, but rather it is digested with a single strand specific exonuclease thereby preventing any subsequent primer binding events (p. 21 1st full paragraph of arguments).
>
> The reply assert that there is therefore no description in Scheele for preserving the single stranded segment that is generated by the treatment of an RNA primer with RNase H so that more binding and extension events can take place to generate more copies of the nucleic acid (p. 22 1st paragraph). The reply asserts that teachings of Scheele would not allow such an event to take place because the exonuclease is present at the same time as the RNase H, eliminating the primer biding site that would be needed for binding of a second RNA primer (p. 22 1st paragraph). The reply asserts that the step before the RNase H step entails the inactivation of Pol I, which would render the polymerase

incapable of using the RNA primer to make a second copy (p. 22 1st paragraph)…

…..The reply seems to be asserting that claim 112 requires the same primer binding even to occur, however, the claim has not been limited to such a step. Specifically step d requires digestion with RNase H wherein the removal allows for another DNA molecule to be produced. Herein in the instant case, Scheele et al. teaches the amplification via PCR. Scheele et al teaches a sample of ds cDNA is prepared and added to its RNA primer with its DNA tail extension intact and excess RNA primers and excess oligo (dT). Taq and dNTPs are further added. The mixture is then subjected to PCR and then RNase is added. As such Scheele et al. teach that multiple copies of the DNA molecule of interest are produced. The applicant seems to be asserting that the main difference between Scheele et al. and the claimed method is that the claim method requires the addition of RNase H before multiple copies are produced. However, the claim has a larger breadth than this limitation. Step d only requires the digestion of the substrate with RNase H so that the substrate is capable of another primer binding event to occur. The wherein clause does not limit the last step to a positive recitation of removing the RNA segment with RNase H and then producing another DNA molecule by performing steps a-d.

(C) The reply points to column 2 (19) of the '926 application which states "the regeneration of a primer binding site thereby allows a new priming event to occur and the production of more than one copy of said specific nucleic acid (p. 22 2nd paragraph). The reply asserts that a priming event would be considered to be both the binding of a primer to its complementary site as well as extension and consequently a second priming event results in synthesis of a second copy (p. 22 2nd paragraph). The reply asserts that although Scheele describes the use of an RNA primer, he teaches away for this priming event because he carries out a step that prevents second binding events after RNase H digestion (p. 22 3rd paragraph)……..

As stated above, the claims are not limited to production of the second copy of the nucleic acid by the steps of a-d. Rather the claims are limited to production of multiple copies of the nucleic acid and the digestion with RNase H. Herein it is the position of the examiner that the claims are not limited to such a recitation of positive active steps. Further it is not clear where the applicant is pointing to with the recitation of

the '926 application as this application does not appear to be either the application number of the instant application or the application number of Scheele et al. However it is noted that the claims are not limited to particular priming events, but rather the wherein clause merely requires that the removal step will have the property or the ability of another primer binding event to occur.

(D) The reply asserts that step d has been amended to specifically claim other binding events by claiming "digesting said substrate with RNase H to remove said ribonucleic acid segment of said extended primer, wherein said removal allows another priming event to occur with said nucleic acid of interest" (p. 22 last paragraph –p. 23 1st paragraph)…

…As stated above, the claims are not limited to production of the second copy of the nucleic acid by the steps of a-d. Rather the claims are limited to production of multiple copies of the nucleic acid and the digestion with RNase H. Herein it is the position of the examiner that the claims are not limited to such a recitation of positive active steps. Further it is not clear where the applicant is pointing to with the recitation of the '926 application as this application does not appear to be either the application number of the instant application or the application number of Scheele et al. However it is noted that the claims are not limited to particular priming events, but rather this wherein statement does not require any active process to occur, it merely requires that the removal step will have the property or the ability of another primer binding event to occur.

Before substantively responding to the rejection, Applicants wish to point out that US 2005/0123926 also referred in the previous response as the '926 application is the published version of the instant application. Applicants intentions in using the published version was strictly for the purpose of making it easier to cite appropriate passages of the disclosure that are relevant to points being discussed in our Response. Applicants will again make use of the published application for references in this response and hope that it will now be clearly understood that references to the "926 application" will refer to passages taken from the U.S. 2005/0123926 application. For examiner's reference, the '926 application is attached hereto as Appendix A.

Applicants note, with respect to the Office Action, that reference is made to Scheele concerning the use of RNase to digest RNA moieties in a primer (cited as being present in column 3 and 4 as well as Figure 5) and the use of PCR to make multiple copies (cited as being in column 8). However, even though Scheele describes the use of RNase H for digestion and the use of PCR would involve another priming event take place, there is no description in Scheele of a priming event being due to the removal of a primer segment by RNase H as required by the claim. In contrast, a subsequent priming event is described even in the Office Action as being allowed by the use of denaturation: "Therefore in a PCR cycle **the resultant dsDNA of step c would be denatured such that the strands of DNA produced** which are identical to the DNA of interest **can be used in the PCR cycle to produce more copies of the DNA of interest**." (Page 4 of the Office Action with emphasis added). There is no description in Scheele itself that RNase H digestion "allows another priming event to occur" as required for step (d) of the method of claim 112. Since RNase H does not perform this function in the method of Scheele, they are forced to substitute another method, thermocycling, for regeneration of a primer binding site. As Applicants have noted in the previous response, there is a teaching away from the use of RNase H removal to generate a new priming site since as seen in Figure 5, the next step after removal of the $r(G)_n$ primer segment by RNase H, is a further removal of the homopolymeric $d(C)_n$ segment by T4 DNA polymerase; quite clearly the this step will not allow the $d(C)_n$ segment to be used again for a priming event. It isn't a question that the RNase digestion in claim 112 is allowing the "same primer binding event to occur" (page 12 of the Office Action), but rather that RNase H digestion in Scheele is not responsible for allowing any particular primer binding events to take place and only denaturation is used to allow further priming events. As such, Applicants are unable to see how the series of RNase H and T4 DNA polymerase digestions described in Scheele is a series of events that would justify a teaching of "wherein said removal allows another priming event to occur". No connection between RNA removal and allowing the template to be used for another priming event is present in Scheele. These are independent events where a) RNase H is used to generate a single-stranded "tail' that is subsequently removed by T4 DNA polymerase and b) PCR carries out a series of subsequent primer

binding events but neither a) or b) is dependent on the other. If a primer is then used in PCR reaction in the remaining double-stranded portion, this would take place in the double-stranded portion that would be totally independent of whether there was removal of the homopolymeric segments by RNase H +T4 DNA polymerase or not. This is also true in the context raised in the Office Action concerning the use of PCR cited from column 8 of the Scheele reference where part of the method is described in column 9, lines 2-5 of Scheele as follows:

> .....the RNA.DNA homopolymeric extensions on each ds cDNA molecule so generated can be removed with RNase H and T4 DNA polymerase , as described in the Example given above. (emphasis added).

Clearly even in conjunction with PCR, the RNase H removal is not being used to allow another primer binding event *per se* but is part of a "trimming" process dedicated only to removing the primer binding site used for the first priming and extension event.

Applicants assert that claim 112 is clearly distinguished from the Scheele reference. However, in order to further prosecution and to emphasize features of the present method that is distinct from those of Scheele, step (d) and the "thereby" portion of claim 112 to emphasize the connection between RNase digestion and subsequent primer binding events that results in production of more copies as follows:

> (d) digesting said substrate with RNase H to remove said ribonucleic acid segment of said extended primer, wherein said removal allows another priming event to occur with **said one or more specific polynucleotide primers comprising at least one ribonucleic acid segment complementary to** said DNA molecule of interest,
>
> thereby producing multiple copies of said nucleic acids of interest by means of said steps (c) and (d).

Applicants wish to point out that claim 112 as amended requires that (a) another priming binding event takes place because(b) an RNase digestion takes place. Further, as written, only the "specific polynucleotide primers comprising at least one ribonucleic acid segment" are present in the mixture provided in step (b) of claim 112 and consequently they would be the only primers available for "another primer binding event". This gives a particular reason for the removal by RNase H since the digestion of the RNA segment would then allow the regeneration of the same primer binding site

that was used in step (c) and thereby another priming event should automatically undergo the same extension and subsequent RNase H removal described for the first primer binding event since the mixture provided in the contacting step (b) contains the nucleic acid producing catalyst and RNase H. Applicants believe that it is abundantly clear that regeneration of a primer binding sequence allows a repetition of the previous set of reactions. For instance, [0018] of the '926 application states that:

> By removing such sequences, a primer binding site is regenerated thereby allowing a new priming event to occur and production of more than one copy of the specific nucleic acid.

and in [0019] of the '926 application it states:

> The regeneration of a primer site thereby allows a new priming event to occur and the production of more than one copy of said specific nucleic acid.

and in [0020] of the '926 application it states that:

> The regeneration of a primer site thereby allows a new priming event to occur and the production of more than one copy of said specific nucleic acid.

and in [0054] of the '926 application it is stated that:

> Under conditions where the primer is an oligonucleotide or copolymer, the primer can be removed from its cognate binding site using specific enzymatic digestion (e.g. RNase H, restriction enzymes and other suitable nucleases) such that another primer can bind and initiate synthesis. This can be used as a system for the multiple initiation of the synthesis of polynucleotide or oligonucleotide product.

Thus, it can be seen that the regeneration of a primer binding site is directly responsible for a subsequent binding and extension of another primer. As such, it is clearly self-evident that in a mixture of DNA templates and primers comprising RNA segments, the binding and extension of one such primer followed by digestion with RNase H will regenerate a primer binding site that would be used for binding from the pool of unextended primers comprising RNA segments which would again be extended by the nucleic acid catalyst present in step (b). This other copy of the primer with an RNA segment hybridized to the DNA template is the same substrate that was used in the

initial RNase H digestion and should undergo the same binding, extension and digestion process, thereby regenerating another primer binding site and so on.

Claims 113-120 ultimately depend from claim 112. Therefore, arguments made with respect to claim 112 would apply to these claims as well.

2.2 Claims 123-130

The Office Action on page 7 with respect 123 states:

> Scheele teaches a method of adding excess primer (e.g. multiple copies of the copolymer primer) (Column 8 lines 58-60). Scheele et al. teaches that once the dsDNA is generated RNase H is used to remove the RNA primer (Column 9 lines 1-5). Therefore once the RNA segment from the primer is removed the template is used to amplify another target strand by using another copolymer primer.

The Office Action on page 16 further states with respect to claim 123:

> (G) The reply asserts that with reference to page 7 and comments on claim 123, the same remarks, which have been stated with regard to claim 112, are maintained (p. 24 1st full paragraph). The reply asserts that on p. 8 of the office action there is a more complete description of the adapted PCR method of Scheele, but that is an alternative methodology wherein the use of RNase H has been eliminated and the primer removal is carried out by the PCR (p. 24 3rd paragraph). The reply asserts that the RNase H step is only added by Scheele after amplification in conjunction with exonuclease and it is not responsible itself for any amplification but only trimming the PCR product (p. 24 3rd full paragraph). The reply asserts that therefore Scheele actually teaches that once a target strand has been amplified by another polymer, the RNA segment from the primer is removed and that there is no description in the Scheele reference of any amplification taking place after the RNase H step (p. 25 1st paragraph)...
>
> ...Again these arguments are drawn to the interpretation of step d. It is noted that the wherein clause does not require any active process to occur, it merely requires that the removal step will have the property or the ability of another primer binding event to occur. Step d does not specifically require amplification after digesting, but rather requires digestion and multiple copies to be produced. Therefore the

> teachings of Scheele et al. provide all the limitations of the positive active steps of the claims.

Applicants, in response, again assert that there is no connection in Scheele between RNase removal of an RNA segment from an extended primer to provide for a subsequent priming event. RNase H is not used by Scheele to bind another primer, but rather denaturation is used to bind primers in subsequent steps. In order to more distinctly claim the subject matter of the invention, step (d) and the "thereby" phrase of claim 123 has been amended to read as follows:

> (d) removing said RNA segment of said extended copolymer primer from said template by digesting with RNase H to bind another **copy of said** copolymer primer to said template and initiate synthesis, **so that multiple initiation of** polynucleotide or oligonucleotide synthesis **occurs**.

As amended, claim 123 clearly describes that a segment is removed in order to bind a primer and initiate synthesis.

With regard to remarks on page 7 of the Office Action that "Scheele teaches a method of adding excess primer (e.g. multiple copies of the copolymer primer)", Applicants note that Scheele (column 8 lines 58-60) states:

> to a sample of ds cDNA prepared by the method of the invention with its RNA /DNA extension still intact, is added excess RNA primer (identical to the RNA primer used to generate the original ds cDNA) and excess oligo(dT) .....the mixture is subjected to an appropriate number of PCR temperatures\ cycles in a PCR machine..." (emphasis added)

It would follow that for the RNA primer/DNA tail extension to be intact in this embodiment of the method of Scheele, there must be an obligatory omission of the RNase H step previously described by Scheele. Removal of the RNA primer segments from templates is then carried out by thermocycling and not by RNase H treatment.

In contrast, amended claim 123 clearly states that the RNase H digestion is the mechanism by which another copy of the copolymer is able to bind and initiate another extension event. The provision of a supply of copolymer primers in contacting step (b) provides a pool from which unextended copolymer primers can bind to a template after an extended copolymer primer is digested with RNase H.

Claims 124-130 depend from claim 123. Therefore, arguments made with respect to claim 123 would apply to claims 124-130 as well.

2.3 Claims 143-145 and 149-150

The Office Action on pages 8 and 9 assert with respect to claim 143:

> With regard to Claim 143, step a, Scheele teaches providing a first DNA strand (e.g. a DNA molecule of interest) (Column 3 lines 25).
>
> With regard to step b, Scheele teaches contacting the DNA with dNTPs (e.g. nucleic acid precursors) (Column 4 lines 20-22). Scheele teaches a primer comprising an RNA segment and a DNA segment by teaching that some of the nucleotide sequence of the primer is an RNA sequence therefore the primer sequence would include DNA (e.g. a copolymer primer) (Column 3 lines 33-40). Scheele teaches a method of adding E. coli DNA polymerase I (e.g. effective amount of nucleic acid producing catalyst) (Column 4 lines 14-15). Scheele teaches a method of adding RNase H (Column 4 lines 25).
>
> With regard to step c, Scheele teaches a method of carrying out synthesis in the presence of the RNA primer to generate a polynucleotide comprising an RNA/DNA hybrid (Column 3 lines 30-40 and Figure 5). The instant specification does not define isostatic conditions of temperature, buffer and ionic strength. Scheele teaches combining the primer and the DNA molecule in a reagent solution at a particular heat with buffers that have a particular ionic strength to produce at least one copy of the DNA molecule by extension of the primer (Column 8 lines 1-15). Therefore Scheele teaches a method of allowing the mixture to react under isostatic condition of temperature, buffer, and ionic strength.
>
> With regard to step d, Scheele teaches a method of digesting the substrate with RNase H to remove the ribonucleic acid segment of the extended primer (Column 3 lines 30-40, column 4 lines 25-28, and Figure 5). Scheele teaches that the method can by adapted to permit amplification of the sample of dsDNA by PCR methodology (Column 8 lines 58-60). Scheele teaches that the reagents of the RNA primer, DNA target, dNTPs and Taq are placed into a PCR machine with the appropriate number of PCR temperature cycles (Column 8 lines 65-69). Therefore in a PCR cycle the resultant dsDNA of step c would be

denatured such that multiple copies of the DNA could be copied from the DNA of interest.

Applicants traverse the rejection. With regard to comments on page 8 of the Office Action concerning Claim 143, the presence of the term "thereby" at the end of step (d) should be sufficient to point out that producing more than one copy is a consequence of regenerating a primer binding site by RNase H and therefore distinguishable from Scheele. However, since Applicants have already included the limitation "under isostatic conditions" in the claim, Applicants are clarifying the significance of this limitation by amending the preamble to stipulate it is an isostatic process overall and steps (c) and (d) of the claim have been amended to more clearly emphasize that step (d) is not a separate step and takes place as a consequence of allowing said mixture to react under isostatic conditions. Applicants wish to specifically point out that the phrase "and thereby producing" has been amended to recite "to produce". Applicants have also amended the nature of the binding site descriptions. The sections that are amended read as follows:

> 143. (currently amended) An **isostatic** *in vitro* process for producing more than one copy....
>
> (c) allowing said mixture to react under isostatic conditions of temperature, buffer and ionic strength, thereby **(i)** producing at least one copy of said DNA molecule by extension of said primer and **(ii)** removing ribonucleotides from said ribonucleic acid segment using said RNase H, to regenerate a primer binding site on said DNA molecule, to render said primer binding site available for another primer binding event **to take place at said regenerated primer binding site to produce** more than copy of said DNA molecule **under isostatic conditions**.

Applicants further wish to clarify as discussed during the interview between Applicants' representative, Cheryl H. Agris and the Examiner on June 16, 2010 that the term "isostatic" means conditions under a given temperature, pH, ionic strength as stated in the first sentence of paragraph [0061]:

> Modification of the primers could either increase or decrease the binding of primer to the target at a given pH, temperature and ionic strength, in other words, at isostatic conditions of pH, temperature and ionic strength, e.g., ionic salt.

It is believed that the above amendments should clearly demarcate differences between the present invention and the method of Scheele with respect to claim 143. Claims 144-145 and 149-150 ultimately depend from claim 143. Thus, arguments made with respect to claim 143 would apply to these claims as well.

In view of the amendments of claims 112, 123 and 143 and the above arguments, Applicants assert that the rejections under 35 USC §102 have been overcome. Therefore, Applicants respectfully request that these rejections be withdrawn.

3. The Rejections Under 35 USC §103

Two grounds of rejections under 35 USC §103 were made and are set forth below.

3.1 The Rejection of Claims 146-148

Claims 146-148 have been rejected under 35 USC §103(a) as being unpatentable over Scheele (US Patent 5162209 November 10, 1992) ("Scheele") in view of Schuster et al. (US Patent 5169766 December 8, 1992) ("Schuster"). The Office Action with respect to Schuster states on pages 17 and 18:

> (I) The reply assets that with regard to p. 13 of the previous office action and step d of claim 146, as previously discussed the claim has been amended to disclose that the RNase H is used to produce more than one copy which is not taught by Scheele et al. (p. 27 2nd paragraph).....
>
> With regard to claim 146, step c of the claim includes producing at least one copy of the DNA molecule by extension of the primer under isostatic conditions and then step d is removing the ribonucleotides from the RNA segment by reverse transcriptase to regenerate the primer binding site on said DNA molecule and thereby produce more than one copy of the DNA molecule. The applicant seems to be asserting that the production of the multiple DNA copies is preformed in step d after the addition of the reverse transcriptase, however, based on the teaches of step c it appears that the DNA copies are produced in step c. The way the claim is written encompasses an interpretation that the DNA copies are made in step c (e.g. the PCR step of Scheele et al) and then the RNA is removed to regenerate

> the primer binding site (e.g. the step of RNase H of Scheele et al). Therefore although the primer binding site is available to another binding event, the multiple copies are actually produced in step c. Step d does not require that another copy of the DNA molecule be produced after the addition of the reverse transcriptase.

Applicants, in response, take issue with the viewpoint expressed in the Office Action that DNA copies are made in step (c) and that this is the same as the PCR step of Scheele, since claim 146 explicitly states that the reaction of step (c) takes place under isostatic conditions. This would intrinsically exclude a thermocycling process such as PCR. Applicants would also state that the regeneration of a primer binding site and making it available thereby produces more than one copy. If it was thereby produced, the action required for it must have taken place. Furthermore, claim 146 has been amended to describe the entire process as being isostatic in nature and as such does not encompass a step that includes the thermocycling steps of PCR described by Scheele regardless of how one interprets the various steps of the claim.

The Office Action further asserts with respect to Scheele on pages 18 and 19:

> (J) The reply asserts with regard to p. 13 and the comments concerning claim146 the Scheele primer is considered to be complementary to the DNA of interest because the DNA of interest includes the added tail and for purposes of being described by claim 148, the Scheele primer is not considered in the office action to be complementary to the DNA of interest since it is now defined as not including the added tail, as such the reply asserts that there is inconsistent definition provided by the office action .....
>
> .....The reply seems to be asserting that the primers of Scheele et al have been characterized by the examiner as both homopolymeric and comprising noncomplementary sequences. However, this is not the case for Scheele et al. Rather Scheele et al teaches a primer that comprises a RNA segment (Column 3 lines 33-40) and it includes a portion that is complementary to an oligonucleotide tail added to the 3' end of the target DNA template (Column 3 lines 25-35). Therefore the primer would comprise a sequence complementary to a distinct sequence of the DNA molecule of interest. Herein in Scheele it would be the RNA segment which is complementary.

> Further it would comprise a portion which is noncomplementary to the DNA of interest. The 3' end is not complementary to the DNA molecule of interest but rather complementary to the tail portion which is added to the DNA. Therefore the primers of Scheele are taught to be partially complementary to a distinct sequence of the DNA molecule and partially noncomplementary...

In response, Applicants assert that the primers or structures of Scheele would not encompass subject matter recited in claims 146-148. Applicants wish to clarify that claim 148 does not say "sequences that are non-complementary to a portion of said DNA molecule" but rather they are described as "non-complementary to said DNA molecule". Applicants note that in [0056] of the instant application, "substantially noncomplementary" is defined as follows:

> In another aspect of the invention, the specific nucleic acid primers are not substantially complementary to one another, having for example, no more than five complementary base-pairs in the sequences therein.

In contrast, in Scheele, the primers are as conceded in the Office Action noncomplementary **to a portion** of the DNA sequence of interest.

The Office Action with respect to the obviousness rejection states on page 22:

> Therefore it would be prima facie obvious to one of ordinary skill in the art to modify the method of Scheele et al. to replace the step of adding a polymerase and RNase H to the nucleic acid sample for a step of adding reverse transcriptase with RNase H activity as taught by Schuster et al. with a reasonable expectation of success. The ordinary artisan would be motivated to replace the step of adding a polymerase and RNase H to the nucleic acid sample for a step of adding reverse transcriptase with RNase H activity as taught by Schuster et al. because Schuster et al. teaches that if an enzyme with RNase H activity is used it is possible to omit a separate RNase H digestion step (Column 8 lines 17-24). Therefore the use of reverse transcriptase with RNase H activity would allow the ordinary artisan to perform the method of Scheele et al. with a reduced number of method steps because only reverse transcriptase with RNase activity must be added to the target to initiate

transcription rather than a polymerase and RNase H and thereby allow for a quicker production of DNA molecules.

Applicants respectfully disagree with the assertion that the substitution of the reverse transcriptase of Schuster into the method of Scheele would be obvious. In Applicants view, the substitution with reverse transcriptase would not have a high likelihood of success. Applicants note that there are two polymerases used by Scheele. T4 DNA polymerase is used for "trimming" a single-stranded tail and polymerases that are insensitive to denaturation conditions are used for PCR. However, Applicants note that T4 DNA polymerase carries out trimming by the action of its exonuclease activity, whereas the reverse transcriptase of Schuster has no such activity. Therefore, reverse transcriptase can't be used for "trimming". Secondly, the reverse transcriptase of Schuster permanently loses all activity under conditions used for DNA denaturation and consequently cannot be used for PCR. As such, substitution of reverse transcriptase for either of the polymerases of Scheele will not allow his methods to be carried out. In addition, as noted above, claim 144 has been amended to describe the entire method as being isostatic in nature which will not allow the use of PCR as described by Scheele for making multiple copies.

The Office Action further states on pages 23 and 24 with respect to the obviousness rejection:

> With regard to claim 146, step c of the claim includes producing at least one copy of the DNA molecule by extension of the primer under isostatic conditions and then step d is removing the ribonucleotides from the RNA segment by reverse transcriptase to regenerate the primer binding site on said DNA molecule and thereby produce more than one copy of the DNA molecule. The applicant seems to be asserting that the production of the multiple DNA copies is preformed in step d after the addition of the reverse transcriptase, however, based on the teaches of step c it appears that the DNA copies are produced in step c. The way the claim is written encompasses an interpretation that the DNA copies are made in step c (e.g. the PCR step of Scheele et al) and then the RNA is removed to regenerate the primer binding site (e.g. the step of RNase H of Scheele et al). Therefore although the primer binding site is available to another binding event, the multiple copies are actually produced in step c. Step d does not require that another

copy of the DNA molecule be produced after the addition of the reverse transcriptase.

In response, as noted above, step (c) is carried out under isostatic conditions, which precludes the use of PCR. Furthermore, Applicants note that step (d) of claim 146 has been amended to recite:

> d) **removing ribonucleotides from said ribonucleic acid segment using said reverse transcriptase**, to regenerate a primer binding site on said DNA molecule, to render said primer binding site available for another primer binding event **to produce more than one copy of said DNA molecule under isostatic conditions.**

As amended, claim 146 now recites that reverse transcriptase is used to regenerate a primer binding site on said DNA molecule, to render said primer binding site available for another primer binding event to produce more than one copy of said DNA molecule.

Applicants note that claims 147 and 148 depend from claim 146. Therefore, arguments made with respect to claim 146 would apply to theses claims as well.

In view of the amendment of claim 146 and the above arguments, Applicants assert that the rejection of claims 146-148 under 35 USC §103 have been overcome. Therefore, Applicants respectfully request that the rejections be withdrawn.

3.2 The Rejection of Claims 133-140

Claims 133-140 have been rejected under 35 USC §103(a) as being unpatentable over Schuster et al. (US Patent 5169766 December 8, 1992) in view of Vary et al. (US Patent 4851331). The Office Action states on pages 25 and 26 with respect to Schuster:

> It is noted that Schuster et al. does teaches the removal of the RNA segments and therefore teaches the regeneration of the primer binding site. Schuster et al teaches destroying RNA with RNase H to produce the first DNA double strand copy (Figure 2). Schuster et al. teaches that RNA is transcribed and that the process can be continued to amplify multiple copies of the RNA molecule of interest (Figure 2).
>
> Therefore Schuster et al. teaches using RNase H to generate a primer binding site on a said copy and that more that one copy is produced. As such Schuster teaches all the required limitations of the claims.

The Office Action with respect to the combination of Schuster and Vary states on pages 24 and 25:

> It would have been prima facie obvious to one of ordinary skill in the art at the time of filing to modify the method of Schuster et al. to use a RNA/DNA primer as taught by Vary et al, in place of the DNA primer used to transcribe the RNA to cDNA. The ordinary artisan would be motivated to use a DNA primer with an end of ribonucleotide in order to have a more active elongation of the template region using E. coli DNA polymerase I. Vary et al. teaches that when using a primer-dependent DNA polymerase of eukaryotic origin primers having a 3' terminal ribonucleotide rather having a 3' terminal deoxynucleotide are more active (Column 9 lines 65-69 and column 10 lines 1-10).

In response, Applicants note that the method of Schuster has no description of digestion of extended primers and only describes the digestion of RNA transcripts (which are primer independent). Secondly with regard to the suggestion that Schuster in combination with Vary would include the use of primers with RNA at the 3' end, there are two steps in Schuster where primer extension is carried out. When RNA is the template, a first cDNA copy is made by primer extension and then the template is digested with RNase H. If a primer is used in this step that has RNA moieties at its 3' end, then the RNA segment of this primer would not be a substrate for RNase H since it is base-paired with the complementary RNA template. The second step where primer extension is carried out by Schuster is when the 1st cDNA copy is used as a template to make a double-stranded molecule. In this step, there is no description of the use or even utility for RNase H described in Schuster. Schuster only describes digestion of molecules that comprise RNA transcripts/1st cDNA copies. In response to the statement on page 25/26 of "to use a RNA/DNA primer as taught by Vary et al., in place of the DNA primer used to transcribe the RNA to cDNA", assuming that it was intended to mean "the DNA primer used to **reverse** transcribe the RNA to cDNA", there would be no removal of the RNA segment from the primer as required by step (e). As such, there is no description of (e) "removing said ribonucleic acid segment of said extended primers with RNase H" even when Vary is combined with Schuster.

Applicants wish to further point out that claim 133 has been amended to recite in step (e):

> (e) removing said ribonucleic acid **segment** of said extended primers with RNase H from **said first DNA copy** of the double-stranded **nucleic acid** produced in step (d) to generate a primer binding site on said second copy of (d) to render said primer binding site available for subsequent primer binding and extension events **to produce** more than one copy of said RNA molecule of interest.

As noted above, in contrast to claim 133, step (e), Schuster teaches that the whole transcript is removed in a primer independent process. Thus adding the primer of Vary would not result in the method of the present in invention.

In view of the above arguments and the amendment of claim 133, Applicants assert that the rejection of claims 133-140 under 35 USC §103 has been overcome. Therefore, Applicants respectfully request that the rejection be withdrawn.

4. Conclusion

Applicants assert that the claims are in condition for allowance. The Examiner is invited to contact the undersigned at (914) 712-0093 if there are any questions regarding this application or response.

Respectfully submitted,

Dated: June 23, 2010

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APPENDIX A

US 20050123926A1

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(54) IN VITRO PROCESS FOR PRODUCING MULTIPLE NUCLEIC ACID COPIES

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(21) Appl. No.: 10/713,183

(22) Filed: Nov. 14, 2003

Related U.S. Application Data

(60) Continuation of application No. 10/260,031, filed on Jun. 6, 2003, which is a continuation of application No. 09/302,816, filed on Mar. 3, 1998, which is a division of application No. 08/182,621, filed on Jan. 13, 1994, now abandoned.

Publication Classification

(51) Int. Cl.[7] C12Q 1/68; C12P 19/34

(52) U.S. Cl. 435/6; 435/91.2

(57) ABSTRACT

This invention provides inter alia an in vitro process for producing multiple specific nucleic acid copies in which the copies are produced under isostatic conditions, e.g., temperature, buffer and ionic strength, and independently of any requirement for introducing an intermediate structure for producing the copies. In other aspects, the invention provides in vitro processes for producing multiple specific nucleic acid copies in which the products are substantially free of any primer-coded sequences, such sequences having been substantially or all removed from the product to regenerate a primer binding site, thereby allowing new priming events to occur and multiple nucleic acid copies to be produced. This invention further provides a promoter-independent non-naturally occurring nucleic acid construct that produces a nucleic acid copy or copies without using or relying on any gene product that may be coded by the nucleic acid construct. Another aspect of this invention concerns a protein-nucleic acid construct in the form of a conjugate linked variously, e.g., covalent linkage, complementary nucleic acid base-pairing, nucleic acid binding proteins, or ligand receptor binding. Further disclosed in this invention is an in vivo process for producing a specific nucleic acid in which such a protein-nucleic acid construct conjugate is introduced into a cell. A still further aspect of the invention relates to a construct comprising a host promoter, second promoter and DNA sequence uniquely located on the construct. The host transcribes a sequence in the construct coding for a different RNA polymerase which after translation is capable of recognizing its cognate promoter and transcribing from a DNA sequence of interest in the construct with the cognate promoter oriented such that it does not promote transcription from the construct of the different RNA polymerase.

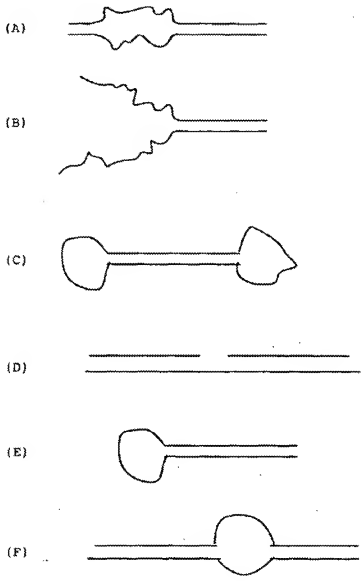


Figure 1 (A-F)

Construct Forms Comprising at Least one Single-Stranded Region

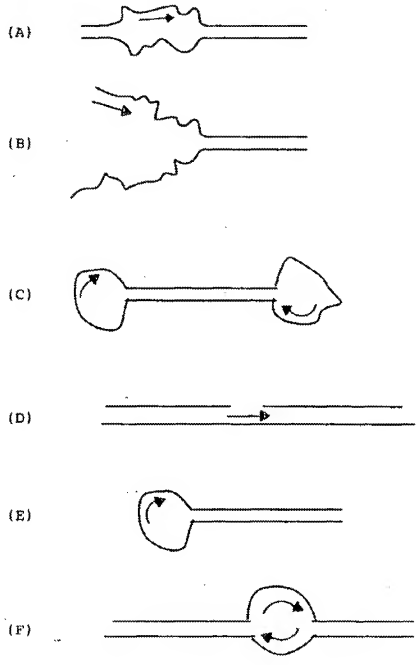


Figure 2 (A-F)

Functional Forms of the Construct

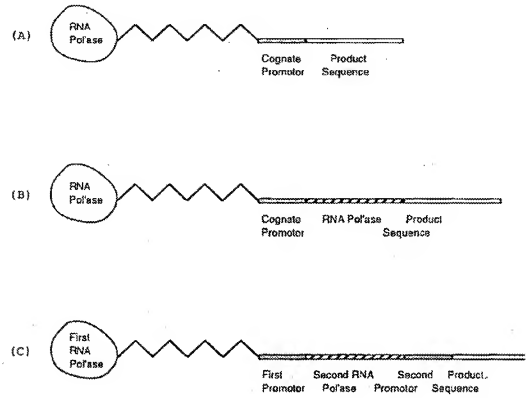


Figure 3 (A-C)

Three Constructs with an RNA Polymerase Covalently Attached to a Transcribing Cassette

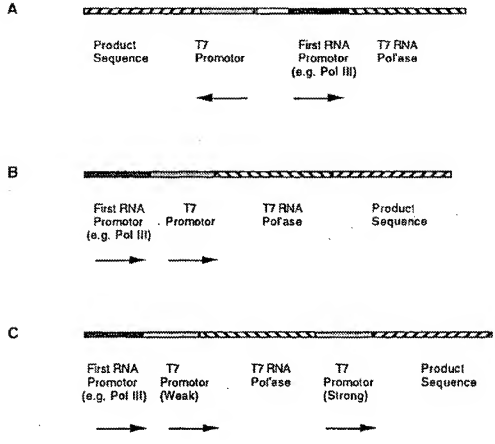


Figure 4 (A-C)

Three Constructs with Promoters for Endogenous RNA Polymerase

M13mp18. Seq Length: 7250

1.	AATGCTACTA	CTATTAGTAG	AATTGATGCC	ACCTTTTCAG	CTCGCGCCCC	
51.	AAATGAAAAT	ATAGCTAAAC	AGGTTATTGA	CCATTTGCGA	AATGTATCTA	
101.	ATGGTCAAAC	TAAATCTACT	CGTTCGCAGA	ATTGGGAATC	AACTGTTACA	
151.	TGGAATGAAA	CTTCCAGACA	CCGTACTTTA	GTTGCATATT	TAAAACATGT	
201	TGAGCTACAG	CACCAGATTC	AGCAATTAAG	CTCTAAGCCA	TCCGCAAAAA	
251	TGACCTCTTA	TCAAAGGAG	CAATTAAAGG	TACTCTCTAA	TCCTGACCTG	
301.	TTGGAGTTTG	CTTCCGGTCT	GGTTCGCTTT	GAAGCTCGAA	TTAAAACGCG	
351.	ATATTTGAAG	TCTTTCGGGC	TTCCTCTTAA	TCTTTTTGAT	GCAATCCGCT	
401.	TTGCTTCTGA	CTATAATAGT	CAGGGTAAAG	ACCTGATTTT	TGATTTATGG	
451.	TCATTCTCGT	TTTCTGAACT	GTTTAAAGCA	TTTGAGGGGG	ATTCAATGAA	
501.	TATTTATGAC	GATTCCGCAG	TATTGGACGC	TATCCAGTCT	AAACATTTTA	
551.	CTATTACCCC	CTCTGGCAAA	ACTTCTTTTG	CAAAAGCCTC	TCGCTATTTT	
601.	GGTTTTTATC	GTCGTCTGGT	AAACGAGGGT	TATGATAGTG	TTGCTCTTAC	
651.	TATGCCTCGT	AATTCCTTTT	GGCGTTATGT	ATCTGCATTA	GTTGAATGTG	
701.	GTATTCCTAA	ATCTCAACTG	ATGAATCTTT	CTACCTGTAA	TAATGTTGTT	
751.	CCGTTAGTTC	GTTTTATTAA	CGTAGATTTT	TCTTCCCAAC	GTCCTGACTG	
801.	GTATAATGAG	CCAGTTCTTA	AAATCGCATA	AGGTAATTCA	CAATGATTAA	
851.	AGTTGAAATT	AAACCATCTC	AAGCCCAATT	TACTACTCGT	TCTGGTGTTC	
901.	TCGTCAGGGC	AAGCTTATT	CACTGAATGA	GCAGCTTTGT	TACGTTGATT	
951.	TGGGTAATGA	ATATCCGGTT	CTTGTCGAAG	ATTACTCTTG	ATGAAGGTCA	
1001	GCCAGCCTAT	GCGCCTGGTC	TGTACACCGT	TCATCTGTCC	TCTTTCAAAG	
1051	TTGGTCAGTT	CGGTTCCCTT	ATGATTGACC	GTCTGCGCCT	CGTTCCGGCT	
1101	AAGTAACATG	GAGCAGGTCG	CGGATTTCGA	CACAATTTAT	CAGGCGATGA	
1151	TACAAATCTC	CGTTGTACCTT	TGTTTCGCGC	TTGGTATAAT	CGCTGGGGGT	
1201	CAAAGATGAG	TGTTTTAGTG	TATTCTTTCG	CCTCTTTCGT	TTTAGGTTGG	

Figure 5

M13mp18 Nucleic Acid Sequence

1251 TGCCTTCGTA GTGGCATTAC GTATTTTACC CGTTTAATGG AAACTTCCTC

1301 ATGAAAAAGT CTTTAGTCCT CAAAGCCTCT GTAGCCGTTG CTACCCTCGT

1351 TCCGATGCTG TCTTTCGCTG CTGAGGGTGA CGATCCCGCA AAAGCGGCCT

1401 TTAACTCCCT GCAAGCCTCA GCGACCGAAT ATATCGGTTA TGGTGGGCG

1451 ATGGTTGTTG TCATTGTCGG CGCAACTATC GGTATCAAGC TGTTTAAGAA

1501 ATTCACCTCG AAAGCAAGCT GATAAACCGA TACAATTAAA GGCTCCTTTT

1551 GGAGCCTTTT TTTTTGGAGA TTTTCAACGT GAAAAAATTA TTATTCGCAA

1601 TTCCTTTAGT TGTTCCTTTC TATTCTCACT CCGCTGAAAC TGTTGAAAGT

1651 TGTTTAGCAA AACCCCATAC AGAAAATTCA TTTACTAACG TCTGGAAAGA

1701 CGACAAAACT TTAGATCGTT ACGCTAACTA TGAGGGTTGT CTGTGGAATG

1751 CTACAGGCGT TGTAGTTTGT ACTGGTGACG AAACTCAGTG TTACGGTACA

1801 TGGGTTCCTA TTGGGCTTGC TATCCCTGAA AATGAGGGTG GTGGCTCTGA

1851 GGGTGGCGGT TCTGAGGGTG GCGGTTCTGA GGGTGGCGGT ACTAAACCTC

1901 CTGAGTACGG TGATACACCT ATTCCGGGCT ATACTTATAT CAACCCTCTC

1951 GACGGCACTT ATCCGCCTGG TACTGAGCAA AACCCGCTA ATCCTAATCC

2001 TTCTCTTGAG GAGTCTCAGC CTCTTAATAC TTTCATGTTT CAGAATAATA

2051 GGTTCCGAAA TAGGCAGGGG GCATTAACTG TTTATACGGC CACTGTTACT

2101 CAAGGCACTG ACCCCGTTAA AACTTATTAC CAGTACACTC CTGTATCATC

2151 AAAAGCCATG TATGACGCTT ACTGGAACGG TAAATTCAGA GACTGCGCTT

2201 CAAGGCACTG ACCCCGTTAA AACTTATTAC CAGTACACTC CTGTATCATC

2151 AAAAGCCATG TGCCTCAACC TCCTGTCAAT GCTGGCGCGG GCTCTGGTGG

2201 TCCATTCTGG CTTTAATCAA GATCCATTCG TTTGTGAATA TCAAGCCAA

2251 TCGTCTGACC TGCCTCAACC TCCTGTCAAT GCTGGCGCGG GCTCTGGTGG

2301 TGGTTCTGGT GGCGGCTCTG AGGGTGGTGG CTCTGAGGGT GGCGGTTCTG

2351 AGGGTGGCGG CTCTGAGGGA GGCGGTTCCG GTGGTGGCTC TGGTTCCGGT

2401 GATTTTGATT ATGAAAAGAT GGCAAACGCT AATAAGGGGG CTATGACCGA

2451 AAATGCCGAT GAAAACGCGC TACAGTCTGA CGCTAAAGGC AAACTTGATT

Figure 5

2501 CTGTCGCTAC TGATTACGGT GCTGCTATCG ATGGTTTCAT TGGTGACGTT

2551 TCCGGCCTTG CTAATGGTAA TGGTGCTACT GGTGATTTTG CTGGCTCTAA

2601 TTCCCAAATG GCTCAAGTCG GTGACGGTGA TAATTCACCT TTAATGAATA

2651 ATTTCCGTCA ATATTTACCT TCCCTCCCTC AATCGGTTGA ATGTCGCCCT

2701 TTTGTCTTTA GCGCTGGTAA ACCATATGAA TTTTCTATTG ATTGTGACAA

2751 AATAAACTTA TTCCGTGGTG TCTTTCGCGT TCTTTTATAT GTTGCCACCT

2801 TTATGTATGT ATTTTCTACG TTTGCTAACA TACTGCGTAA TAAGGAGTCT

2851 TTATCATGCC AGTTCTTTTG GGTATTCCGT TATTATTGCG TTTCCTCGGT

2901 TTCCTTCTGG TAACTTTGTT CGGCTATCTG CTTACTTTTC TTAAAAAGGG

2951 CTTCGGTAAG ATAGCTATTG CTATTTCATT GTTTCTTGCT CTTATTATTG

3001 GGCTTAACTC AATTCTTGTG GGTTATCTCT CTGATATTAG CGCTCAATTA

3051 CCCTCTGACT TTGTTCAGGG TGTTCAGTTA ATTCTCCCGT CTAATGCGCT

3101 TCCCTGTTTT TATGTTATTC TCTCTGTAAA GGCTGCTATT TTCATTTTTG

3151 ACGTTAAACA AAAAATCGTT TCTTATTTGG ATTGGGATAA ATAATATGGC

3201 TGTTTATTTT GTAACTGGCA AATTAGGCTC TGGAAAGACG CTCGTTAGCG

3251 TTGGTAAGAT TCAGGATAAA ATTGTAGCTG GGTGCAAAAT AGCAACTAAT

3301 CTTGATTTAA GGCTTCAAAA CCTCCCCGAA GTGGGGAGGT TCGCTAAAAC

3351 GCCTCGCGTT CTTAGAATAC CGGATAAGCC TTCTATATCT GATTTGCTTG

3401 CTATTGGGCG CCGTAATGAT TCCTACGAATG AAAATAAAAA CGGCTTGCTT

3451 GTTCTCGATG AGTGGGGTAC TTGGTTTAAT ACCCGTTCTT GGAATGATAA

3501 GGAAAGACAG CCGATTATTG ATTGGTTTCT ACTGCTCGT AAATTAGGAT

3551 GGGATATTAT TTTTCTTGTT CAGGACTTAT CTATTGTTGA TAAACAGGCG

3601 CGTTCTGCAT TAGCTGAACA TGTTGTTTAT TGTCGTCGTC TGGACACAAT

3651 TACTTTACCT TTTGTCGGTA CTTTATATTC TCTTATTACT GGCTCGAAAA

3701 TGCCTCTGCC TAAATTACAT GTTGGCGTTG TTAAATATGG CGATTCTCAA

3751 TTAAGCCCTA CTGTTGAGCG TTGGCTTTAT ACTGGTAAGA ATTTGTATAA

3801 CGCATATGAT ACTAAACAGG CTTTTTCTAG TAATTATGAT TCCGGTGTTT

Figure 5

M13mp18 Nucleic Acid Sequenc

3851 ATTCTTATTT AACGCCTTAT TTATCACACG GTCGGTATTT CAAACCATTA

3901 AATTTAGGTC AGAAGATGAA ATTAACTAAA ATAATATTGA AAAAGTTTTC

3951 TCGCGTTCTT TGTCTTGCGA TTGGATTTGC ATCAGCATTT ACATATAGTT

4001 ATATAACCCA ACCTAAGCCG GAGGTTAAAA AGGTAGTCTC TCAGACCTAT

4051 GATTTTGATA AATTCACTAT TGACTCTTCT CAGCGTCTTA ATCTAAGCTA

4101 TCGCTATGTT TTCAAGGATT CTAAGGGAAA ATTAATTAAT AGCGACGATT

4151 TACAGAAGCA AGGTTATTCA CTCACATATA TTGATTTATG TACTGTTTCC

4201 ATTAAAAAAG GTAATTCAAA TGAAATTGTT AAATGTAATT AATTTTGTTT

4251 TCTTGATGTT TGTTTCATCA TCTTCTTTTG CTCAGGTAAT TGAAATGAAT

4301 AATTCGCCTC TGCGCGATTT TGTAACTTGG TATTCAAAGC AATCAGGCGA

4351 AATCCGTTATT GTTTCTCCCG ATGTAAAAGG TACTGTTACT GTATATTCAT

4401 CTGACGTTAA ACCTGAAAAT CTACGCAATT TCTTTATTTC TGTTTTACGT

4451 GCTAATAATT TTGATAATGGT TGGTTCAATT CCTTCCATAA TTCAGAAGTA

4501 TAATCCAAAC AATCAGGATT ATATTGATGA ATTGCCATCA TCTGATAATC

4551 AGGAATATGA TGATAATTCC GCTCCTTCTG GTCGTTTCTT TGTTCCGCAA

4601 AATGATAATG TTACTCAAAC TTTTAAAATT AATAACGTTC GGGCAAGGA

4651 TTTAATACGA GTTGTCGAAT TGTTTGTAAA GTCTAATACT TCTAAATCCT

4701 CAAATGTATT ATCTATTGAC GGCTCTAATC TATTAGTTGT TAGTGCTCCT

4751 AAAGATATTT TAGATAACCT TCCTCAATTC CTTTCTACTG TTGATTTGCC

4801 AACTCACCAG ATATTGATTG AGGGTTTGAT ATTTGAGGTT CAGCAGGGTG

4851 ATGCTTTAGA TTTTTCATTT GCTGCTGGCT CTCAGCGTGG CACTGTTGCA

4901 GGCGGTGTTA ATACTGACGC CCTCAGCTCT GTTTTATCTT CTGCTGGTGG

4951 TTCGTTCGGT ATTTTTAATG GCGATGTTTT AGGGCTATCA GTTCGCGCAT

5001 TAAAGACTAA TAGCCATTCA AAAATATTGT CTGTGCCACG TATTCTTACG

5051 CTTTCAGGTC AGAAGGGTTC TATCTCTGTT GGCCAGAATG TCCCTTTTAT

5101 TAAAGACTAA TAGCCATTCA AAAATATTGT CTGTGCCACG TATTCTTACG

5151 CGATTGAGCG TCAAAATGTA GGTATTTCCA TGAGCGTTTT TCCTGTTGCA

Figure 5

M13mp18 Nucleic Acid Sequence

5201 ATCGCTGGCG GTAATATTGT TCTGGATATT ACCAGCAAGG CCGATAGTTT
5251 GAGTTCTCT ACTCAGGCAA GTGATGTTAT TACTAATCAA AGAAGTATTG
5301 CTACAACGGT TAATTTGCGT GATGGACAGA CTCTTTTACT CGGTGGCCTC
5351 ACTGATTATA AAAACACTTC TCAAGATTCT GGCGTACCGT TCCTGTCTAA
5401 AATCCCTTTA ATCGGCCTCC TGTTTAGCTC CCGCTCTGAT TCCAACGAGG
5451 AAAGCACGTT ATACGTGCTC GTCAAAGCAA CCATAGTACG CGCCCTGTAG
5501 CGGCGCATTA AGCGCGGCGG GTGTGGTGGT TACGCGCAGC GTGACCGCTA
5551 CACTTGCCAG CGCCCTAGCG CCCGCTCCTT TCGCTTTCTT CCCTTCCTTT
5601 CTCGCCACGT TCGCCGGCTT TCCCCGTCAA GCTCTAAATC GGGGGCTCCC
5651 TTTAGGGTTC CGATTTAGTG CTTTACGGCA CCTCGACCCC AAAAAACTTG
5701 ATTTGGGTGA TGGTTCACGT AGTGGGCCAT CGCCCTGATA GACGGTTTTT
5751 CGCCCTTTGA CGTTGGAGTC CACGTTCTTT AATAGTGGAC TCTTGTTCCA
5801 AACTGGAACA ACACTCAACC CTATCTCGGG CTATTCTTTT GATTTATAAG
5851 GGATTTTGCC GATTTCGGAA CCACCATCAA ACAGGATTTT CGCCTGCTGG
5901 GGCAAACCAG CGTGGACCGC TTGCTGCAAC TCTCTCAGGG CCAGGCGGTG
5951 AAGGGCAATC AGCTGTTGCC CGTCTCGCTG GTGAAAAGAA AAACCACCCT
6001 GGCGCCCCAT ACGCAAACCG CCTCTCCCCG CGCGTTGGCC GATTCATTAA
6051 TGCAGCTGGC ACGACAGGTT TCCCGACTGG AAAGCGGGCA GTGAGCGCAA
6101 CGCAATTAAT GTGAGTTAGC TCACTCATTA GGCACCCCAG GCTTTACACT
6151 TTATGCTTCC GGCTCGTATG TTGTGTGGAA TTGTGAGCGG ATAACAATTT
6201 CACACAGGAA ACAGCTATGA CCATGATTAC GAATTCGAGC TCGGTACCCG
6251 GCGATCCTCT AGAGTCGACC TGCAGGCATG CAAGCTTGGC ACTGGCCGTC
6301 GTTTTACAAC GTCGTGACTG GGAAAACCCT GGCGTTACCC AACTTAATCG
6351 CCTTGCAGCA CATCCCCTTT CGCCAGCTGG CGTAATAGCG AAGAGGCCCG
6401 CACCGATCGC CCTTCCCAA CAGTTGCGCA GCCTGAATGG CGAATGGCGC
6451 TTTGCCTGGT TTCCGGCACC AGAAGCGGTG CCGGAAAGCT GGCTGCAGTG
6501 CGATCTTCCT GAGCCCGATA CGGTCGTCGT CCCCTCAAAC TGGCAGATGC

Figure 5

M13mp18 Nucleic Acid Sequence

6551 ACGGTTACGA TGCGCCCATC TACACCAACG TAACCTATCC CATTACGGTC

6601 AATCCGCCGT TTGTTCCCAC GGAGAATCCG ACGGGTTGTT ACTCGCTCAC

6651 ATTTAATGTT GATGAAAGCT GGCTACAGGA AGGCCAGACG CGAATTATTT

6701 TTGATGGCGT TCCTATTGGT TAAAAAATGA GCTGATTTAA CAAAAATTTA

6751 ACGCGAATTT TAACAAAATA TTAACGTTTA CAATTTAAAT ATTTGCTTAT

6801 ACAATCTTCC TGTTTTTGGG GCTTTTCTGA TTATCAACCG GGGTACATAT

6851 GATTGACATG CTAGTTTTAC GATTACCGTT CATCGATTCT CTTGTTTGCT

6901 CCAGACTCTC AGGCAATGAC CTGATAGCCT TTGTAGATCT CTCAAAAATA

6951 GCTACCCTCT CCGGCATGAA TTTATCAGCT AGAACGGTTG AATATCATAT

7001 TGATGGTGAT TTGACTGTCT CCGGCCTTTC TCACCCTTTT GAATCTTTAC

7051 CTACACATTA CTCAGGCATT GCATTTAAAA TATATGAGGG TTCTAAAAAT

7101 TTTTATCCTT GCGTTGAAAT AAAGGCTTCT CCCGCAAAAG TATTACAGGG

7151 TCATAATGTT TTTGGTACAA CCGATTTAGC TTTATGCTCT GAGGCTTTAT

Figure 5

M13mp18 Nucleic Acid Sequence

COMPLEMENTARY TO M_{13}

POSITION	5' 3'	POSITION	
645	AGCAACACTATCATA	631	$M_{13}/1$
615	ACGACGATAAAAACC	601	$M_{13}/2$
585	TTTTGCAAAAGAAGT	571	$M_{13}/3$
555	AATAGTAAAATGTTT	541	$M_{13}/4$
525	CAATACTGCGGAATG	511	$M_{13}/5$
495	TGAATCCCCCTCAAA	481	$M_{13}/6$
465	AGAAAACGAGAATGA	451	$M_{13}/7$
435	CAGGTCTTTACCCTG	421	$M_{13}/8$
405	AGGAAAGCGGATTGC	391	$M_{13}/9$
375	AGGAAGCCCGAAAGA	361	$M_{13}/10$

COMPLEMENTARY TO SS PHAGE DNA

POSITION	5' 3'	POSITION	
351	ATATTTGAAGTCTTT	366	$M_{13}/11$
371	TCTTTTTGATGCAAT	386	$M_{13}/12$
391	CTATAATACTCAGGG	406	$M_{13}/13$
411	TGATTTATGGTCATT	426	$M_{13}/14$
431	GTTTAAAGCATTTGA	446	$M_{13}/15$
451	TATTTATGACGATTC	466	$M_{13}/16$
471	TATCCAGTCTAAACA	486	$M_{13}/17$
491	CTCTGGCAAAACTTC	506	$M_{13}/18$
511	TCGCTATTTTGGTTT	526	$M_{13}/19$
531	AAACGAGGGTTATGA	546	$M_{13}/20$

Figure 6

Primers for Nucleic Acid Production Derived from M13mp18 Sequence

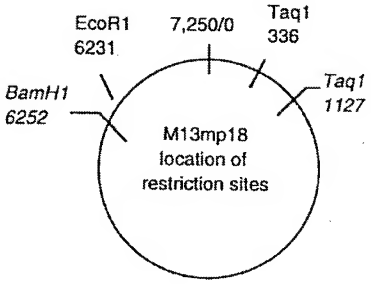
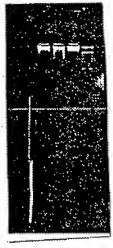


Figure 7

Appropriate M13mp18 Restriction Sites

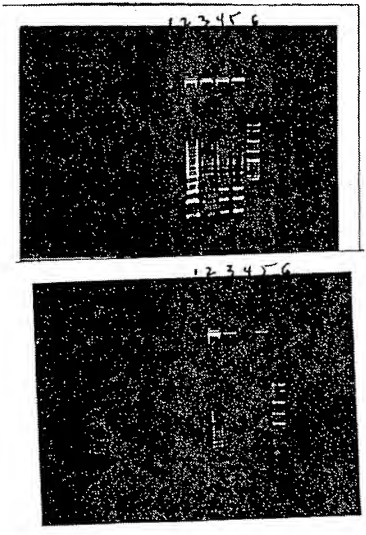


Lane 1: from calf thymus + Taq digested mp18 amplification reaction
Lane 2: from Taq digested mp18 amplification reaction
Lane 3: from calf thymus amplification reaction
Lane 4: øX174 HinfI size marker

Figure 8

Lane 1: no template
Lane 2: mp18 template, phosphate buffer
Lane 3: MspI/pBR322 size marker
Lane 4: mp18 template, MOPS buffer

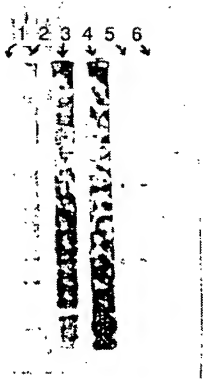
Figure 9



Top= (+) Template
Bottom= (-) Template

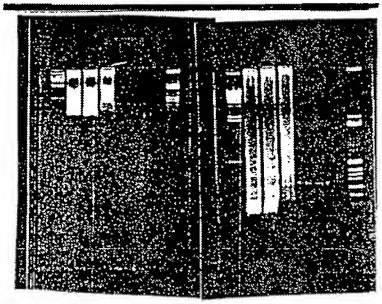
Lane 1: phosphate buffer
Lane 2: MES
Lane 3: MOPS
Lane 4: DMAB
Lane 5: DMG
Lane 6: pBR322/MspI size marker

Figure 10



Lane 1: DMAB buffer, no template
Lane 2: DMAB buffer, mp18 template
Lane 3: DMG buffer, no template
Lane 4: DMG buffer, mp18 template
Lane 5: No reaction
Lane 6: 200 ng Taq I digested mp18 size marker/positive control

Figure 11



First Time Interval Second Time Interval

Agarose Gel Analysis

Lane 1: lambda Hind III marker
Lane 2: Amp/Untreated
Lane 3: Amp/Kinased
Lane 4: Amp/Kinased/Ligated
Lane 5: PCR/Untreated
Lane 6: PCR/Kinased
Lane 7: PCR/Kinased/Ligated
Lane 8: øX174/Hinf1 marker

Figure 12

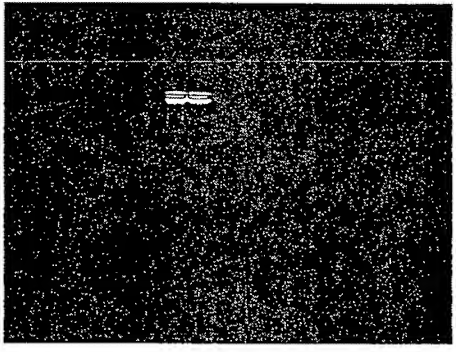
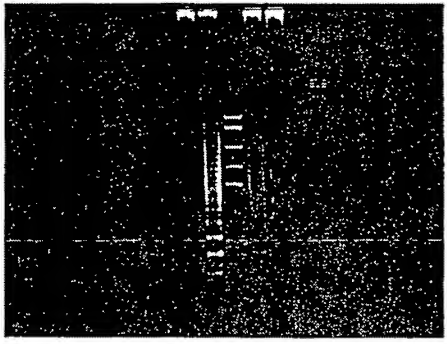


Figure 13



Lane 1: Primers alone
Lane 2: Primers + taq digested M13 DNA
Lane 3: Molecular weight markers
Lane 4: Primers + RNA
Lane 5: Primers alone
Lane 6: M13 digested DNA
Buffer was dimethyl amino glycine, pH 8.6

Figure 14

Lane 1: Primers alone
Lane 2: Primers + taq digested M13 DNA
Lane 3: Molecular weight markers
Lane 4: Primers + RNA
Lane 5: Primers alone
Lane 6: M13 digested DNA
Buffer was dimethyl amino glycine, pH 8.6

Figure 15

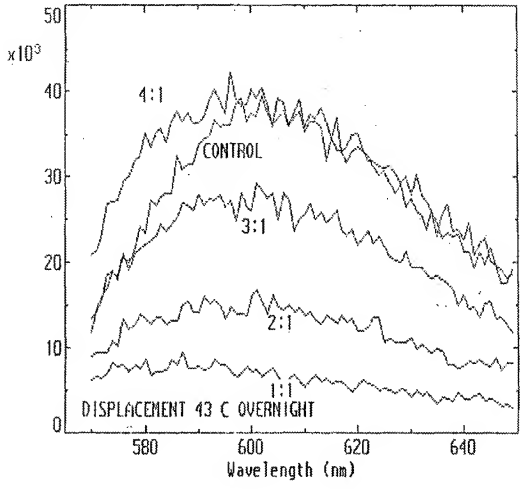

50
x10^3
40
30
20
10
0
4:1
CONTROL
3:1
2:1
1:1
DISPLACEMENT 43 C OVERNIGHT
580
600
620
640
Wavelength (nm)

Figure 16

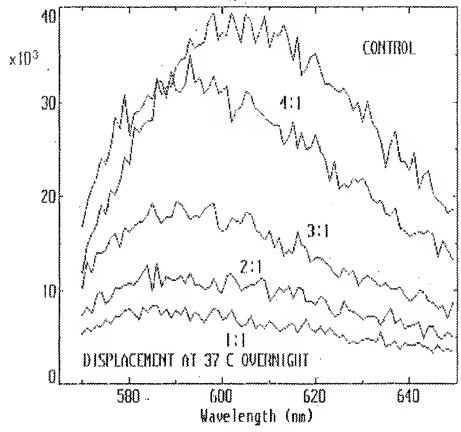

x10^3
40
30
20
10
0
CONTROL
4:1
3:1
2:1
1:1
DISPLACEMENT AT 37 C OVERNIGHT
580
600
620
640
Wavelength (nm)

Figure 17

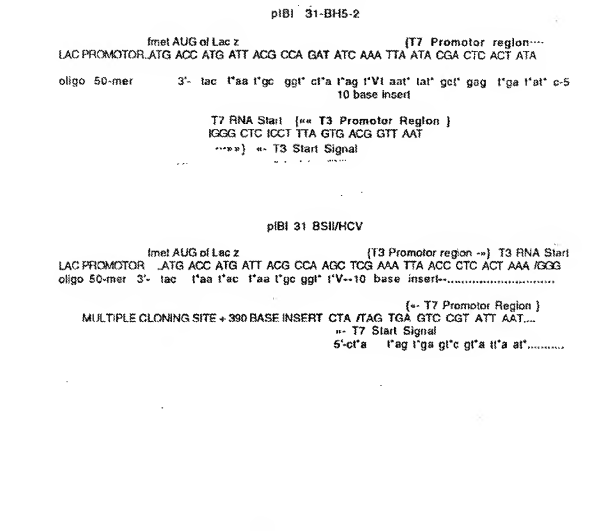

pIBI 31-BH5-2
fmet AUG of Lac z
{T7 Promotor region
LAC PROMOTOR..ATG ACC ATG ATT ACG CCA GAT ATC AAA TTA ATA CGA CTC ACT ATA
oligo 50-mer
3'- tac t*aa t*gc ggt* ct*a t*ag t*Vt aat* tat* gct* gag t*ga t*at* c-5
10 base insert
T7 RNA Start {«« T3 Promotor Region }
IGGG CTC ICCT TTA GTG ACG GTT AAT
«-» } «- T3 Start Signal
pIBI 31 BSII/HCV
fmet AUG of Lac z
{T3 Promotor region -» } T3 RNA Start
LAC PROMOTOR ..ATG ACC ATG ATT ACG CCA AGC TCG AAA TTA ACC CTC ACT AAA /GGG
oligo 50-mer 3'- tac t*aa t*ac t*aa t*gc ggt* t*V--10 base insert--
{«- T7 Promotor Region }
MULTIPLE CLONING SITE + 390 BASE INSERT CTA /TAG TGA GTC CGT ATT AAT....
«- T7 Start Signal
5'-ct*a t*ag t*ga gt*c gt*a tt*a at*.....

Figure 18

IN VITRO PROCESS FOR PRODUCING MULTIPLE NUCLEIC ACID COPIES

FIELD OF THE INVENTION

[0001] This invention relates to the field of in vitro and in vivo production of nucleic acid production and to nucleic constructs and protein-nucleic acid conjugates for use in such production.

[0002] All patents, patent publications, scientific articles, and videocassettes cited or identified in this application are hereby incorporated by reference in their entirety in order to describe more fully the state of the art to which the present invention pertains.

BACKGROUND OF THE INVENTION

[0003] Current methodology cited heretofore in the literature relating to amplification of a specific target nucleic acid sequence in vitro essentially involve 2 distinct elements:

[0004] 1 repeated strand separation or displacement or a specific "intermediate" structure such as a promoter sequence linked to the primer or introduction an assymetric restriction site not originally present in the nucleic acid target; followed by

[0005] 2 production of nucleic acid on the separated strand or from an "intermediate" structure.

[0006] Separation can be accomplished thermally or by enzymatic means. Following this separation, production is accomplished enzymatically using the separated strands as templates

[0007] Of the established amplification procedures, Polymerase Chain Reaction (PCR) is the most widely used. This procedure relies on thermal strand separation, or reverse transcription of RNA strands followed by thermal dissociation. At least one primer per strand is used and in each cycle only one copy per separated strand is produced. This procedure is complicated by the requirement for cycling equipment, high reaction temperatures and specific thermostable enzymes. (Saiki, et al., Science 230:1350-1354 (1985); Mullis and Faloona, Methods-in Enzymology 155: 335-351 (1987); U.S. Pat. Nos. 4,683,195 and 4,883,202).

[0008] Other processes, such as the Ligase Chain Reaction (LCR) (Backman, K., European Patent Application Publication No. 0 320 308; Landegren, U., et al. Science 241 1077 (1988); Wu, D. and Wallace, R. B. Genomics 4 560 (1989); Barany, F. Proc. Nat. Acad. Sci. USA 88 189 (1991)), and Repair Chain Ligase Reaction (RLCR) or Gap Ligase Chain Reaction (GLCR) (Backman, K. et al. (1991) European Patent Application Publication No. 0 439 182 A, Segev, D. (1991) European Patent Application Publication No. 0 450 594) also use repeated thermal separation of the strands and each cycle produces only one ligated product. These procedures are more complicated than PCR because they require the use of an additional thermostable enzyme such as a ligase.

[0009] More complicated procedures are the Nucleic Acid Sequence Based Amplification (NASBA) and Self Sustained Sequence Reaction (3SR) amplification procedures. (Kwoh, D. Y. et al., Proc Nat Acad Sci., USA, 86:1173-1177 (1989); Guatelli, J. C. et al., 1990 Proc Nat Acad Sci., USA 87:1874-1878 (1990) and the Nucleic Acids Sequence Based Amplification (NASBA) (Kievits,T., et al J. Virol. Methods 35:273-286 (1991); and Malek, L. T., U.S. Pat. No. 5,130, 238). These procedures rely on the formation of a new "intermediate" structure and an array of different enzymes, such as reverse transcriptase, ribonuclease H, T7 RNA polymerase or other promotor dependant RNA polymerases and they are further disadvantaged by the simultaneous presence of ribo- and deoxyribonucleotide triphosphates precursors.

[0010] For the intermediate construct formation, the primer must contain the promotor for the DNA dependent RNA polymerase. The process is further complicated because the primer is, by itself, a template for the RNA polymerase, due to its single-stranded nature.

[0011] The last of the major amplification procedures is Strand Displacement Amplification (SDA) (Walker, G. T. and Schram, J. L., European Patent Application Publication No. 0 500 224 A2; Walker, G. T. et al. European Patent Application No. 0 543 612 A2; Walker, G. T., European Patent Application Publication No. 0 497 272 A1, Walker, G. T. et al., Proc Natl Acad Sci USA 89:392-396 (1992); and Walker, G. T. et al., Nuc Acids Res. 20:1691-1696 (1992). The intermediate structure of this procedure is formed by the introduction of an artificial sequence not present in the specific target nucleic acid and which is required for the asymetric recognition site of the restriction enzyme. Again this procedure involves more than one enzyme and the use of thio nucleotide triphosphate precursors in order to produce this assymetric site necessary for the production step of this amplification scheme.

[0012] The random priming amplification procedure (Hartley, J. L., U.S Pat No 5,043,272) does not relate to specific target nucleic acid amplification.

[0013] Probe amplification systems have been disclosed which rely on either the amplification of the probe nucleic acid or the probe signal following hybridization between probe and target. As an example of probe amplification is the Q-Beta Replicase System (Qβ) developed by Lizardi and Kramer and their colleagues. Qβ amplification is based upon the RNA-dependent RNA polymerase derived from the bacteriophage Qβ. This enzyme can synthesize large quantities of product strand from a small amount of template strand, roughly on the order of 10^6 to 10^9 (million to billion) increases. The Qβ replicase system and its replicatable RNA probes are described by Lizardi et al., "Exponential amplification of recombinant RNA hybridization probes," *Biotechnology* 6:1197-1202 (1988); Chu et al., U.S. Pat No. 4,957, 858; and well as by Keller and Manak (*DNA Probes*, MacMillan Publishers Ltd, Great Britain, and Stockton Press (U.S. and Canada, 1989, pages 225-228). As discussed in the latter, the Qβ replicase system is disadvantaged by non-specific amplification, that is, the amplification of non-hybridized probe material, which contributes to high backgrounds and low signal-to-noise ratios. Such attendant background significantly reduces probe amplification from its potential of a billion-fold amplification to something on the order of 10^4 (10,000 fold). In addition, the Q beta amplification procedure is a signal amplification—and not a target amplification.

[0014] In vivo

[0015] Literature covering the introduction of genes or antisense nucleic acids into a cell or organism is very

extensive (Larrick, J. W. and Burck, K. Gene Therapy Elsevier Science Publishing Co., Inc, New York (1991); Murray, J. A. H. ed Antisense RNA and DNA, Wiley-Liss, Inc., New York (1992)). The biological function of these vectors generally requires inclusion of at least one host polymerase promoter.

[0016] The present invention as it relates to in vitro and in vivo production of nucleic acids is based on novel processes, constructs and conjugates which overcome the complexity and limitations of the above-mentioned documents.

SUMMARY OF THE INVENTION

[0017] The present invention provides an in vitro process for producing more than one copy of a specific nucleic acid in which the process is independent of any requirement for the introduction of an intermediate structure for the production of the specific nucleic acid. The process comprises three steps, including (a) providing a nucleic acid sample containing or suspected of containing the sequence of the specific nucleic acid; (b) contacting the sample with a three component reaction mixture; and (c) allowing the mixture to react under isostatic conditions of temperature, buffer and ionic strength, thereby producing more than one copy of the specific nucleic acid. The reaction mixture comprises: (i) nucleic acid precursors, (ii) one or more specific nucleic acid primers each of which is complementary to a distinct sequence of the specific nucleic acid, and (iii) an effective amount of a nucleic acid producing catalyst.

[0018] In another aspect, the present invention provides an in vitro process for producing more than one copy of a specific nucleic acid in which the products are substantially free of any primer-coded sequences. Such a process comprises the following steps, including (a) providing a nucleic acid sample containing or suspected of containing the sequence of the specific nucleic acid; (b) contacting the sample with a three component mixture (the mixture comprising (i) nucleic acid precursors, (ii) one or more specific polynucleotide primers comprising at least one ribonucleic acid segment each of which primer is substantially complementary to a distinct sequence of the specific nucleic acid, and (iii) an effective amount of a nucleic acid producing catalyst); and (c) allowing the mixture to react under isostatic conditions of temperature, buffer and ionic strength, thereby producing at least one copy of the specific nucleic acid; and (d) removing substantially or all primer-coded sequences from the product produced in step (c). By removing such sequences, a primer binding site is regenerated, thereby allowing a new priming event to occur and producing more than one copy of the specific nucleic acid.

[0019] The present invention also provides an in vitro process for producing more than one copy of a specific nucleic acid in which the products are substantially free of any primer-coded sequences. In the steps of this process, said process comprising a nucleic acid sample containing or suspected of containing the sequence of the specific nucleic acid is provided, and contacted with a reaction mixture. The mixture comprises (i) unmodified nucleic acid precursors, (ii) one or more specific chemically-modified primers each of which primer is substantially complementary to a distinct sequence of said specific nucleic acid, and (iii) an effective amount of a nucleic acid producing catalyst. The mixture thus contacted is allowed to react under isostatic conditions of temperature, buffer and ionic strength, thereby producing at least one copy of the specific nucleic acid. In a further step, substantially or all primer-coded sequences from the product produced in the reacting step is removed to regenerate a primer binding site. The regeneration of a primer binding site thereby allows a new priming event to occur and the production of more than one copy of said specific nucleic acid.

[0020] An additional provision of the present invention is an in vitro process for producing more than one copy of a specific nucleic acid in which the products are substantially free of any primer-coded sequences. In this instance, the process comprises the steps of: (a) providing a nucleic acid sample containing or suspected of containing the sequence of the specific nucleic acid; and (b) contacting the sample with a reaction mixture (the mixture comprising (i) unmodified nucleic acid precursors, (ii) one or more specific unmodified primers comprising at least segment each of which primer comprises at least one non-complementary sequence to a distinct sequence of the specific nucleic acid, such that upon hybridization to the specific nucleic acid, at least one loop structure is formed, and (iii) an effective amount of a nucleic acid producing catalyst). The mixture so formed is allowed to react in step (c) under isostatic conditions of temperature, buffer and ionic strength, thereby producing at least one copy of the specific nucleic acid wherein step is followed by (d) removing substantially or all primer-coded sequences from the product produced in step (c) to regenerate a primer binding site. The regeneration of a primer binding site thereby allows a new priming event to occur and the production of more than one copy of said specific nucleic acid.

[0021] Another embodiment of the present invention concerns a promoter-independent non-naturally occurring nucleic acid construct which when present in a cell produces a nucleic acid without the use of any gene product coded by the construct.

[0022] In yet another embodiment, the present invention provides a conjugate comprising a protein-nucleic acid construct in which the nucleic acid construct does not code for said protein, and which conjugate produces a nucleic acid when present in a cell.

[0023] The present invention also has significant in vivo applications. In one such application, an in vivo process is provided for producing a specific nucleic acid. The in vivo process comprises the steps of (a) providing a conjugate comprising a protein-nucleic acid construct, the conjugate being capable of producing a nucleic acid when present in a cell; and (b) introducing such a conjugate into a cell, thereby producing the specific nucleic acid.

[0024] Another significant aspect of the present invention relates to a construct comprising a host promoter located on the construct such that the host transcribes a sequence in the construct coding for a different RNA polymerase, which after translation is capable of recognizing its cognate promoter and transcribing from a DNA sequence of interest from the construct with the cognate promoter oriented such that it does not promote transcription from the construct of the different RNA polymerase.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] FIG. 1(A-F) depicts various nucleic acid constructs forms contemplated by the invention in which at least one single-stranded region are located therein.

[0026] FIG. 2(A-F) depicts the functional forms of the nucleic acid constructs illustrated in FIG. 1(A-F).

[0027] FIG. 3(A-C) is an illustration of three nucleic acid constructs with an RNA polymerase covalently attached to a transcribing cassette.

[0028] FIG. 4(A-C) illustrates three nucleic acid constructs with promoters for endogenous RNA polymerase.

[0029] FIG. 5 is a nucleic acid sequence for M13mp18.

[0030] FIG. 6 shows the sequence and the positions of the primers derived from M13mp18 which were employed in the presen invention for nucleic acid production.

[0031] FIG. 7 illustrates appropriate restriction sites in M13mp18.

[0032] FIG. 8 is an agarose gel with a lane legead illustrating the experimental results in Example 5 in which amplification of the M13 fragment was carried out in the presence of a large excess (1500 fold) of irrelevant DNA.

[0033] FIG. 9 is an agarose gel with a lane legead illustrating the results in Example 8 in which the effect of variations of reaction conditions on the product obtained in Example 3 was investigated.

[0034] FIG. 10 is an agarose gel with a lane legend that illustrates the results of a qualitative analysis of the effects observed in Example 9 of various buffers on the amplification reaction in accordance with the present invention.

[0035] FIG. 11 is a southern blot (with lane legend) obtained from Example 10 in which two buffers, DMAB and DMG, were separately employed in nucleic acid production.

[0036] FIG. 12 is an agarose gel and lane legend obtained in Example 11 in which the nature of the ends of amplified product was investigated.

[0037] FIG. 13 is an agarose gel obtained in Example 12 in which amplification from non-denatured template was examined.

[0038] FIG. 14 is an agarose gel obtained in Example 13 in which amplification from an RNA template was examined.

[0039] FIG. 15 is a southern blot of the gel obtained in FIG. 14.

[0040] FIG. 16 is a fluorescence spectrum illustrating the results obtained in Example 14 in which the phenomenon of strand displacement using ethidium-labeled oligonucleotides in accordance with the present invention was investigated.

[0041] FIG. 17 is a fluorescence spectrum illustrating the results obtained in Example 15 in which a T7 promoter oligonucleotide 50 mer labeled with ethidium was employed to study its effects on in vitro transcription by T7 and T3 polymerases from an IBI 31 plasmid (pIBI 31-BH5-2) and from a BlueScript II plasmid construct (pBSII/HCV).

[0042] FIG. 18 depicts the polylinker sequences of the IBI 31 plasmid (pIBI 31-BH5-2) and the BlueScript II plasmid constructs (pBSII/HHCV).

DETAILED DESCRIPTION OF THE INVENTION

[0043] The present invention describes novel methods and constructs for production of multiple copies of specific nucleic acid sequences in vitro and in vivo

[0044] One aspect of this invention represents an in vitro process for the production of more than one copy of nucleic acid from specific target nucleic acid (either DNA or RNA) sequences utilizing a biological catalyst, e.g., a DNA polymerase, primer oligonucleotides complementary to sequences (primer sites) in the target nucleic acid. The production process can proceed in the presence of a large excess of other nucleic acids and does not require thermal cycling or the introduction of -specific intermediate constructs such as promoters or assymetric restriction sites, etc.

[0045] More particularly, this invention provides an in vitro process for producing more than one copy of a specific nucleic acid, the process being independent of a requirement for the introduction of an intermediate structure for the production of any such specific nucleic acid. The in vitro production process comprises the steps of: (a) providing a nucleic acid sample containing or suspected of containing the sequence of the specific nucleic acid, (b) contacting the sample with a three component mixture; and (c) allowing the thus-contacted mixture to react under isostatic conditions of temperature, buffer and ionic strength, thereby producing more than one copy of the specific nucleic acid. The three component mixture just alluded will generally comprise (i) nucleic acid precursors, (ii) one or more specific nucleic acid primers each of which is complementary to a distinct sequence of the specific nucleic acid, and (iii) an effective amount of a nucleic acid producing catalyst. In other aspects, the specific nucleic acid may be single-stranded or double-stranded, and may take the form of deoxyribonucleic acid, ribonucleic acid, a DNA:RNA hybrid or a polymer capable of acting as a template for a nucleic acid polymerizing catalyst.

[0046] In addition, the specific nucleic acid can be in solution in which case the above-described in vitro process may further comprise the step of treating the specific nucleic acid with a blunt-end promoting restriction enzyme. Further, isolation or purification procedures can be employed to enrich the specific nucleic acid. Such procedures are well-known in the art, and may be carried out on the specific nucleic acid prior to the contacting step (b) or the reacting step (c). One means of isolation or purification of a nucleic acid involves its immobilization, for example, by sandwich hybridization (Ranki et al., 1983), or sandwich capture. Particularly significant in the latter methodology is the disclosure of Engelhardt and Rabbani, U.S. patent application Ser. No. 07/968,706, filed on Oct. 30, 1992, entitled "Capture Sandwich Hybridization Method and Composition," now allowed, that was published as European Patent Application Publication No. 0 159 719 A2 on Oct. 30, 1985. The contents of the foregoing U.S. patent application is incorporated herein by reference.

[0047] The target nucleic can be be present in a variety of sources. For purposes of disease diagnoses these would

include blood, pus, feces, urine, sputum, synovial fluid, cerebral spinal fluid, cells, tissues, and other sources. The production process can be performed on target nucleic that is present in samples which are free of interfering substances, or the production process can be performed on target nucleic acid separated from the sample. The nucleic acid can be in solution or bound to a solid support. While the replication process can be carried out in the presence of nonrelevant nucleic acids, certain applications may require prior separation of the target sequences. Methods such as sandwich hybridization or sandwich capture referenced above can then be applied to immobilize target sequences. In such instances where sandwich hybridization or sandwich capture is carried out, the above-described in vitro process may further comprise the step of releasing the captured nucleic acid, e.g., by means of a restriction enzyme.

[0048] As described above, the target sequence need not be limited to a double-stranded DNA molecule. Target molecules could also be single stranded DNA or RNA. For example, replication of a single-stranded target DNA could proceed using primers complementary to both the single-stranded DNA target and to the produced complementary sequence. Following the initial synthesis of the complementary sequence DNA, production from this strand would begin. RNA can serve as the template using a DNA polymerase I, e.g., Klenow, which can reverse transcribe under conditions that have been described (Karkas, J. D. et al., Proc Nat Acad Sci U.S.A. 69:398-402 (1972))

[0049] In case the target nucleic acid is double stranded, a restriction digest or sonication, partial endonuclease treatment or denaturation could be employed for the preparation of the target nucleic acid before the onset of amplification.

[0050] An aspect of this invention concerns its use in determining whether a specific target nucleic acid was derived from a living or a deceased organism. To make such a determination, one could in parallel amplify and detect the presence of a specific target DNA or a specific target RNA associated with the genomic makeup of the organism; and thereafter amplify and detect the presence of a specific RNA target associated to the biological function (living function) of the organism which does not survive if the organism is deceased

[0051] The nucleic acid precursors contemplated for use in the present invention are by and large well-known to those skilled in the art. Such precursors may take the form of nucleoside triphosphates and nucleoside triphosphate analogs, or even combinations thereof. More particularly, such nucleoside triphosphates are selected from deoxyadenosine 5'-triphosphate, deoxyguanosine 5'-triphosphate, deoxythymidine 5'-triphosphate, deoxycytidine 5'-triphosphate, adenosine 5'-triphosphate, guanosine 5'-triphosphate, uridine 5'-triphosphate and cytidine 5'-triphosphate, or a combination of any of the foregoing. Such nucleoside triphosphates are widely available commercially, or they may be synthesized by techniques or equipment using commercially available precursors.

[0052] In the case where the nucleic acid precursors comprise nucleoside triphosphate analogs, these are also widely available from a number of commercial sources, or they may be manufactured using known techniques. Such nucleoside triphosphate analogs can be in the form of naturally occurring or synthetic analogs, or both.

[0053] It should not go unrecognized or even unappreciated that the foregoing nucleoside triphosphate and nucleoside triphosphate analogs can be unmodified or modified, the latter involving modifications to the sugar, phosphate or base moieties. For examples of such modifications, see Ward et al., U.S. Pat. No. 4,711,955; Engelhardt et al., U.S. Pat. No. 5,241,060; Stavrianopoulos, U.S. Pat. No. 4,707,440; and Weltmar, Quartin and Engelhardt, U.S. patent application Ser. No. 07/499,938, filed on Mar. 26, 1990, the latter having been disclosed in European Patent Application Serial No. 0 450 370 A1, published on Oct. 9, 1991. The contents of the foregoing U.S. patents and patent application are incorporated by their entirety into the present application

[0054] The primers, one or more, described herein bind to specific sequences on the target nucleic acids and initiate the polymerizing reaction. While oligo deoxynucleotide primers may be preferred, polydeoxynucleotide as well as oligo and polyribonucleotide or nucleotide copolymer primers can be used (Kornberg, A. and T. A. Baker, second edition, 1992, W.H. Freeman and Co. New York; Karkas, J. D., PNAS 69:2288-2291 (1972); and Karkas, J. D. et al., Proc. Natl. Acad. Sci. U.S.A. 69:398-402 (1972)). Thus, the specific nucleic acid primers may be selected from deoxyribonucleic acid, ribonucleic acid, a DNA:RNA copolymer, or a polymer capable of hybridizing or forming a base-specific pairing complex and initiating nucleic acid polymerization. Under conditions where the primer is an oligoribonucleotide or copolymer, the primer can be removed from its cognate binding site using specific enzymatic digestion (e.g., RNase H, restriction enzymes and other suitable nucleases) such that another primer can bind and initiate synthesis. This can be used as a system for the multiple initiation of the synthesis of polynucleotide or oligonucleotide product.

[0055] Modifications, including chemical modifications, in the composition of the primers would provide for several novel variations of the invention. See, for example, U.S. Pat. Nos. 4,711,955; 5,241,060; 4,707,440; and U.S. patent application Ser. No. 07/499,938, supra. For example, substitution of the 3 hydroxyl group of the primer by an isosteric configuration of heteroatoms, e.g., a primary amine or a thiol group, would produce chemically cleavable linkers. In the case of thiol excess of another thiol in the reaction mixture will cleave the phosphorothioate linkers which is formed after the initiation of polymerization, thus allowing the DNA polymerase to reinitiate polymerization with the same primer. Thus, in this variation repeated syntheses can begin from a modified, hybridized primer providing a significant increase in the synthesis of DNA.

[0056] In another aspect of the invention, the specific nucleic acid primers are not substantially complementary to one another, having for example, no more than five complementary base-pairs in the sequences therein.

[0057] In another variation, the primer could contain some noncomplementary sequences to the target, whereupon hybridization would form at least one loop or bubble which could be used as a substrate for a specific endonuclease such that the primer could be removed from the target by enzymatic digestion thus allowing reinitiation. Furthermore, the primer could contain additional sequences noncomplementary to the target nucleic acid. Thus, the specific nucleic acid primers may comprise at least one non-complementary nucleotide or nucleotide analog base, or at least one

sequence thereof. The range of non-complementarity may range in some cases from about 1 to about 200 noncomplementary nucleotide or nucleotide analogs, and in other cases, from about 5 to about 20 nucleotides. Such noncomplementary base sequence or sequences can be linked by other than a phosphodiester bond.

[0058] As used herein, the term "nucleic acid producing catalyst" is intended to cover any agent, biological, physical or chemical in nature, that is capable of adding nucleotides (e.g., nucleoside triphosphates, nucleoside triphosphate analogs, etc.) to the hydroxyl group on the terminal end of a specific primer (DNA or RNA) using a pre-existing strand as a template. A number of biological enzymes are known in the art which are useful as polymerizing agents. These include, but are not limited to *E. coli* DNA polymerase I, Klenow polymerase (a large proteolytic fragment of *E. coli* DNA polymerase I), bacteriophage T7 RNA polymerase, and polymerases derived from thermophilic bacteria, such as *Thermus aquaticus*. The latter polymerase are known for their high temperature insensitivity, and include, for example, the Taq DNA polymerase I. A thermostable Taq DNA polymerase is disclosed in Gelfand et al., U.S. Pat. No. 4,889,818. Preferred as a polymerizing agent in the present invention is the Taq DNA Polymerase I. Many if not all of the foregoing examples of polymerizing agents are available commercially from a number of sources, e.g., Boehringer-Mannheim (Indianapolis, Ind.). Particularly suitable as nucleic acid producing catalysts are DNA polymerase and reverse transcriptase, or both. As used herein, "the effective amount" of the nucleic acid producing catalyst is an art-recognized term reflecting that amount of the catalyst which will allow for polymerization to occur in accordance with the present invention.

[0059] Since the rate and extent of hybridization of the primers is dependent upon the standard conditions of hybridization (Wetmur, J. G. and Davidson, N. J., Mol. Biol. 31:349 (1968)), the concentration and nucleotide sequence complexity of the total primers added to the reaction mixture will directly affect the rate at which they hybridize and accordingly the extent to which they will initiate nucleic acid synthesis. In addition, if the reaction is run under conditions where the guanine triphosphate is replaced by inosine triphosphate or other modified nucleoside triphosphates such that the presence of this modified nucleotide in the product nucleic acid would lower the melting temperature of the product:template double helix, then at any given temperature of the reaction the extent of breathing of the double helix will be increased and the extent of binding of the primers to the target strand will be enhanced.

[0060] Furthermore, primers could displace the strands at the ends of the double stranded target and hybridize with one of the two strands and, this displacement hybridization reaction (or D loop formation reaction) is favored by adding more than one primer molecule. In general, as the total amount of the sequence complexity of the primers complementary to the target nucleic acid is increased a greater nucleic acid production is obtained (see Example 3 below).

[0061] Modification of the primers could either increase or decrease the binding of primer to the target at a given pH, temperature and ionic strength, in other words, at constant conditions of pH, temperature and ionic strength, e.g., ionic salt. Other primer modifications can be employed which would facilitate polymerization from the primer sites, even when the initiation site is within a double helix. For example, once an oligo primer is introduced into a target double stranded nucleic acid molecule, if such an oligo primer is modified with ethidium or any moiety that increases the melting temperature of the double stranded structure formed by the oligo and a target nucleic acid, it forms a relatively more stable single stranded structure because of the nucleotide modifications. This produces a primer initiation site. In fact, the nucleic acid precursors or the specific primers (or both) can be modified by at least one intercalating agent, such as ethidium, in which case it may be useful to carry out an additional step (d) of detecting any product produced in step (c), as set forth above. In such a step where desirable, detection can be carried out by means of incorporating into the product a labeled primer, a labeled precursor, or a combination thereof.

[0062] Another additional aspect of the in vitro process, above-described, is the inclusion of a further step of regenerating one or more specific nucleic acid primers, as described elsewhere in this disclosure, including immediately below.

[0063] As described in the summary of this invention, an in vitro process for multiple nucleic acid production is provided in which the products are substantially free of any primer-coded sequences. In such process, the removing step (d) is carried out by digestion with an enzyme, e.g., ribonuclease H. In one aspect of this invention, the nucleic acid precursors are modified or unmodified in the instance where one or more specific polynucleotide primers are used, the primers comprising at least one ribonucleic acid segment and wherein each primer is substantially complementary to a distinct sequence of the specific nucleic acid. Thus, the specific polynucleotide primers may further comprise deoxyribonucleic acid. In another feature of this particular in vitro process, the specific polynucleotide primers contain a 3'-hydroxyl group or an isoteric configuration of heteroatoms, e.g., nitrogen, sulfur, or both. In addition, the polynucleotide primers in this instance may further comprise from about 1 to about 200 noncomplementary nucleotide or nucleotide analogs.

[0064] In yet a further in vitro process for producing more than one copy of a specific nucleic acid is provided (as described in the summary), the products being substantially free of any primer-coded sequences. In this instance, unmodified nucleic acid precursors are reacted in a mixture with one or more chemically-modified primers each of which is substantially complementary to a distinct sequence of the specific nucleic acid. An effective amount of a nucleic acid producing catalyst is also provided in the mixture. As in the case of the last-described in vitro process, the removing step (d) may be carried out by digestion with an enzyme, e.g., ribonuclease H. The specific chemically modified primers are selected, for example, from ribonucleic acid, deoxyribonucleic acid, a DNA:RNA copolymer, and a polymer capable of hybridizing or forming a base-specific pairing complex and initiating nucleic acid polymerization, or a combination of any of the foregoing. The specific chemically modified primers may contain a 3'-hydroxyl group or an isoteric configuration of heteroatoms, N, S, or both, as described above in other in vitro processes. Further, the specific chemically modified primers can be selected from nucleoside triphosphates and nucleoside triphosphate ana-

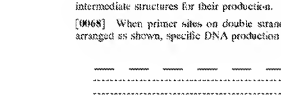
logs, or a combination thereof, wherein at least one of said nucleoside triphosphates or analogs is modified on the sugar, phosphate or base. Also as in other in vitro processes, the specific chemically modified primers may further comprise from about 1 to about 200 noncomplementary nucleotide or nucleotide analogs.

[0065] In still yet another of the in vitro processes for multiple nucleic acid production, described previously in the summary of this invention, unmodified nucleic acid precursors are provided in the mixture and reacting step (c), together with one or more specific unmodified primers comprising at least one segment, each of which primer comprises at least one non-complementary sequence to a distinct sequence of the specific nucleic acid, such that upon hybridization to the specific nucleic acid at least one loop structure is formed. As in the other instances, digestion with an enzyme, e.g., ribonuclease H, may be employed in the removing step (d). In one feature of this process, specific unmodified primers are selected from ribonucleic acid, deoxyribonucleic acid, a DNA:RNA copolymer, and a polymer capable of hybridizing or forming a base-specific pairing complex and initiating nucleic acid polymerization, or a combination of any of the foregoing. Further, the specific unmodified primers may further comprise from about 1 to about 200 noncomplementary nucleotide or nucleotide analogs, in accordance with the present invention.

[0066] The rate of hybridization of the primer to target nucleic acids and, in particular, to target double stranded nucleic acids can be facilitated by binding of the primer with various proteins, e.g., rec A proteins. For example, if the primer is modified with an intercalating agent, e.g., ethidium (or any moiety that increases the melting temperature of the double stranded structure), the addition of this primer to or with a protein such as rec A, either free or bound, would facilitate the introduction of the primer into the double stranded target. (Kornberg and Baker, supra, pages 797-800). This could produce a suitable primer initiation site.

[0067] The arrangement of primer binding sites on the template nucleic acid can be varied as desired. For example, the distance between successive primer binding sites on one strand can also be varied as desired. Also specific primers can be employed that initiate synthesis upstream of the sequence sought to be copied. Under this scenario, multiple copies of nucleic acid are made without successive denaturation or use of other enzymes or the introduction of intermediate structures for their production.

[0068] When primer sites on double stranded DNA are arranged as shown, specific DNA production is increased.



[0069] When the target sequences are substantially covered by their complementary primers, a further increase in the production of multiple copies of nucleic acid is favored due to the increase in initiation points and destabilization of the double stranded template molecule.

[0070] Finally, if an oligo is modified such that it will form a stable hybrid, even in the presence of the complementary nucleic acid strand, then the modified oligo can act as a 'helper' oligo. 'Helper' oligo in this context is defined as a oligo that does not necessarily act as a primer but will accelerate the binding and priming activity of other oligos in the vicinity to the binding site of the 'helper' oligo. Vicinity is here being defined as the location of a nucleotide sequence or the complementary nucleotide sequence close enough to the binding site of the 'helper' oligo to have the rate or extent of hybridization of the primer affected by the binding of the 'helper' oligo. The 'helper' oligo can be modified such that it does not initiate polymerization as for example through the use of a dideoxy 3' terminal nucleotide or other nucleotide with blocked 3' ends. The 'helper' oligo can also be modified in such a manner that the double helix formed by the 'helper' oligo and the target nucleic acid strand or the 'helper' and the complementary strand to the target strand is more stable or has a higher melting temperature than the equivalent double helix of unmodified 'helper' oligo and the target or the strand complementary to the target strand. Such modifications can include halogenation of certain bases, ethenyl pyrimidines (C:C triple bonds, propyne amine derivatives; the addition of ethidium or other intercalating molecules (see Stavrianopoulos and Rubbani, U.S. patent application Ser. No. 07/956,566, filed on Oct. 5, 1992, the contents of which are incorporated herein by reference and which were disclosed in European Patent Application Publication No. 0 231 495 A2, published on Aug. 12, 1987); the supplementation of the oligo with certain proteins that stabilize the double helix and any other treatment or procedure or the addition of any other adduct that serves to stabilize the portion of the double helix with the 'helper' bound or to increase the melting temperature of portion of the double helix with the 'helper' bound.

[0071] In vivo Synthesis of Nucleic Acid

[0072] This invention describes a cassette or nucleic acid construct into which any nucleic acid sequence can be inserted and which can be used as a template for the production of more than one copy of the specific sequence. This cassette is a nucleic acid construct containing a sequence of interest, which within or present within, the cell produces nucleic acid product which is independent or only partially dependent on the host system. The cassette or nucleic acid construct may be characterized as a promoter-independent non-naturally occurring, and in one embodiment comprises double-stranded and single-stranded nucleic acid regions. This construct contains a region in which a portion of the opposite strands are not substantially complementary, e.g., a bubble (even comprising at least one polyT sequence), or loop, or the construct comprises at least one single-stranded region. The construct is composed of naturally occurring nucleotides or chemically modified nucleotides or a synthetic polymer in part or a combination thereof. These structures are designed to provide binding of polymerizing enzymes or primers and the modifications provide for nuclease resistance or facilitate uptake by the target cell.

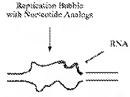
[0073] Referring to the constructs (A-F) depicted in FIG. 3, the single stranded regions described in the constructs will contain coding sequences for nucleic acid primers present in the cell to facilitate initiation points of DNA polymerase in said cell. In the case of RNA polymerase, these constructs constitute promotor independent binding and initiation of RNA polymerase reaction These constructs can be used in

vitro and in vivo for production of nucleic acids. The position of the single stranded region adjacent to the double stranded specific sequence would provide a specific and consistent transcription of these specific sequences, both in vitro and in vivo independent of promotor. The replication (DNA) or transcription (RNA) products of these constructs can be single stranded nucleic acid which could have a sense or antisense function or could be double stranded nucleic acid.

[0074] In **FIG. 13**(A), a large bubble is located in the construct. In **FIG. 13**(B), the two strands are noncomplementary at their ends, and thus do not form a bubble. In **FIG. 13**(C), a double bubble is formed due to noncomplementarity at both ends. In **FIG. 13**(D), a single-stranded region is shown in the middle of the construct leading to a partially single-stranded region (and no bubble formation). **FIG. 13**(E) depicts a bubble at one end of the construct (compare with the two bubbles in the construct shown in **FIG. 13**(C). In **FIG. 13**(F), a single bubble in the middle of the construct is shown. It should be readily appreciated by those skilled in this art that the above-depicted embodiments are representative embodiments not intended to be limiting, particularly in light of the present disclosure.

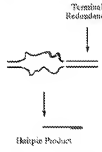
[0075] In vivo these constructs, with a specific primer present in the cell can initiate nucleic acid synthesis. When these primers are RNA, after initiation of nucleic acid synthesis, they can be removed by the action of ribonuclease H, thus vacating the primer binding sequence and allowing other primer molecules to bind and reinitiate synthesis. The cellular nucleic acid synthesizing enzymes can use these constructs to produce copies of a specific nucleic acid from the construct. Shown in **FIG. 14**(A-F) are corresponding illustrations of the constructs in **FIG. 13**(A-F), except that the predirection arrows (points and directions) are indicated.

[0076] These constructs could contain more than one specific nucleic acid sequence which in turn could produce more than one copy of each specific nucleic acid sequence. If two independent nucleic acid products are complementary, then they could hybridize and form multiple copies of a new double stranded construct that could have the properties of the novel construct. Furthermore they could contain promotor sites such as the host promotor therefore serving as an independent nucleic acid production source (the progeny).

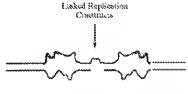


Primer-Dependent DNA Production
Using Nucleic Acid Constructs

[0077] The replication of this structure could result in the production of one strand of DNA product. Several alternative events may occur allowing for the formation of a second complementary strand. For example, a terminal loop could be inserted at the end of the construct such that the single stranded product will code for the synthesis of the complementary strand using the repair enzyme. Constructs can be made that produce single stranded DNA product that has a hairpin loop and therefore, can be used to form a double-stranded product. Alternatively, constructs can be formed that produce nucleic acid in both polarity.

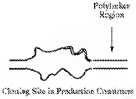


[0078] An alternative approach to the production of double stranded product is to covalently link two constructs that make complementary DNA strands.



Linked Complementary Production Constructs

[0079] The construct can be made to contain a poly linker region into which any sequence can be cloned. The result will be a transient accumulation of expressing genes within the cell to deliver sense, antisense or protein or any other gene product into the target cell.



Cloning Site in Production Constructs

[0080] Other processes within the invention herein described apply to the production of more than one copy of functional genes or antisense DNA or RNA in target cells.

[0081] Production of Primers

[0082] Primers can be produced by several methods. Single-stranded oligonucleotides in the range from between from about 5 to about 100 bases long, and preferably between from about 10 to about 40, and more preferably,

between from about 8 to about 20 nucleotides. These ranges may further vary with optimality between from about 15 to about 30 for bacterial nucleic acid, and optimally between from about 17 to about 35 for eukaryote nucleotides would appear to be appropriate for most applications although it may be desirable in some or numerous instances to vary the length of the primers. Oligonucleotide primers can be most conveniently produced by automated chemical methods. In this way modified bases can be introduced. Manual methods can be used and may in some cases be used in combination with automated methods. All of these methods and automation are known and available in the art.

[0083] In addition nucleic acid primers can be produced readily by the action of T7 RNA polymerase, T3 polymerase, SP6 polymerase or any appropriate DNA or RNA polymerase on DNA templates or RNA templates containing the primer sequences extended from the corresponding RNA polymerase promoter sites or other nucleic acid synthesis start signals.

[0084] Detection of Products

[0085] DNA produced by the invention described herein can be detected by a variety of hybridization methods using homogeneous or non-homogeneous assays. DNA produced in tissues or cells, i.e., in situ, can be detected by any of the practiced methods for in situ hybridization These include, but are not limited to, hybridization of the produced DNA with a nucleic acid probe labeled with a suitable chemical moiety, such as biotin. Probes used for the detection of produced DNA can be labeled with a variety of chemical moieties other than biotin. These include but are not limited to fluorescein, dinitrophenol, ethidium (see, for example, the disclosures of U.S. Pat. Nos. 4,711,955; 5,241,060; and 4,707,440, supra).

[0086] The hybridized, labeled nucleic acid probe can be detected by a variety of means. These include but are not limited to reaction with complexes composed of biotin binding proteins, such as avidin or streptavidin, and color generating enzymes, such as horseradish peroxidase or alkaline phosphatase, which, in the presence of appropriate substrates and chromogens, yield colored products.

[0087] In accordance with this invention, DNA production from target sequences generally requires nucleic acid precursors, e.g., adenosine triphosphate, guanosine triphosphate, thymidine triphosphate and cytosine triphosphate, present in sufficient quantity and concentration in the reaction mixture. In other applications it may be advantageous to substitute one or more of the natural precursors with modified nucleotides. For example, when the invention described herein is being applied to the detection of specific nucleic acid sequences, immobilization of the produced DNA may be desirable. In such an instance, substitution of one or more of the natural nucleoside triphosphate precursors with a modified nucleotide, e.g., biotinylated deoxyuridine triphosphate, in place of thymidine triphosphate, would yield biotin-labeled DNA. The produced DNA could be separated by its affinity for a biotin binding protein, such as avidin, streptavidin or an anti-biotin antibody A variety of nucleoside triphosphate precursors (U.S. Pat. Nos. 4,711,955; 5,241,060, and 4,707,440, supra) labeled with chemical moieties which include, but are not limited to, dinitrophenol and fluorescein, and which can be bound by corresponding antibodies or by other binding proteins can be used in this manner. In other aspects of the invention, the produced DNA can be isotopically labeled by the inclusion of isotopically labeled deoxynucleotide precursors in the reaction mixture.

[0088] Labeled DNA, produced by the invention described herein, can function as probe nucleic acid to be used to detect specific target nucleic acid sequences.

[0089] In certain detection formats the primers may be removed from the reaction mixture by capturing the product through direct capture (Brakel et al., U.S. patent application Ser. No. 07/998,660, filed on Dec. 23, 1992, the contents of which have been disclosed in European Patent Application 0 435 150 A2, published on Jul. 3, 1991; and the contents of which are also incorporated by reference herein), or sandwich capture. (Engelhardt and Rabbani, allowed U.S. patent application Ser No 07/968,706, supra), or by modifying the primers at the 3' end with biotin or immunobiotin without an arm or a very short arm such that the avidin will recognize only the unincorporated primers (single stranded form) but not the incorporated due to the double stranded form and the short length of the arm. Additionally, the primer may be labeled with ethidium or other intercalating moiety. In this condition, the ethidium or other intercalating moiety may be inactivated (Stavrianopoulos, U.S. patent application Ser. No. 07/633,730, filed on Dec. 24, 1990, published as European Patent Application Publication No. 0 492 570 A1 on Jul. 1, 1992; the contents of which are incorporated by reference) in the unhybridized oligo and not in the hybridized oligo/target.

[0090] Another aspect of this invention herein described is to provide for a conjugate of a nucleic acid polymerizing enzyme (RNA polymerase) with a nucleic acid construct said nucleic acid construct contains an initiation site such as a promotor site for the corresponding RNA polymerase which is capable of producing nucleic acid both in vivo and in vitro. The enzyme could be linked directly to the nucleic acid or through a linkage group substantially not interfering with its function or the enzyme could be linked to the nucleic acid indirectly by a nucleic acid bridge or hapten-receptor where the enzyme is biotinylated and the nucleic acid construct contains an avidin or vice versa or when the nucleic acid construct contains sequences for binding proteins such as a repressor and an enzyme linked to said nucleic acid binding protein (U.S. Pat. No. 5,241,060, supra, and Pergolizzi, Stavrianopoulos, Rabbani, Engelhardt, Kline and Olsiewski, U.S. patent application Ser. No. 08/032,769, filed on Mar. 16, 1993, published as European Patent Application Publication No. 0 128 332 A1 on Dec. 19, 1984, the latter having been "allowed" by the European Patent Office, and further incorporated by reference herein).

[0091] Further in regard to the just-described conjugate of the present invention, the protein in one aspect comprises an RNA polymerase or a subunit thereof and the nucleic acid construct contains the corresponding RNA polymerase promoter. The RNA polymerase can be selected from T7, T3 and SP6, or a combination of any of the foregoing. In another embodiment, the protein in the conjugate comprises DNA polymerase or reverse transcriptase and the nucleic acid construct contains at least one sequence complementary to an RNA molecule. The construct can take the form of double-stranded, single-stranded, or even partially single-stranded. Further, the nucleic acid construct in the conjugate may comprise at least one chemically modified nucleotide or

nucleotide analog. The linkages of the protein to the construct are described in the preceding paragraph. The nucleic acid produced by or from this conjugate comprises deoxyribonucleic acid, ribonucleic acid, or combinations thereof, or it may be antisense or sense, or both.

[0092] As described in the summary of the invention, the above-described conjugate may be utilized in an in vivo process for producing a specific nucleic acid. In other aspects of this in vivo process, the construct is further characterized as comprising (independently) at least one promotor, at least one complementary sequence to a primer present in the cell, or codes for the protein in the conjugate, or for a protein other than the protein in the conjugate. The other protein may comprise a nucleic acid polymerase. In the instant where the polymerase comprises an RNA polymerase, the nucleic acid construct may comprise a promoter for the RNA polymerase. Further, the polymerase can be a DNA polymerase or reverse transcriptase.

[0093] (a) Direct Attachment of a Polymerase to the Construct

[0094] For example, if a construct containing a RNA polymerase linked directly or indirectly to a DNA construct or cassette is introduced into a cell, the RNA polymerase will transcribe the nucleic acid in the construct and is completely independent of any host RNA polymerases. Each molecule introduced into a cell will produce multiple copies of a segment of the construct. In the first iteration, the attached polymerase can produce the RNA for the target sequence itself. (See **FIG. 3(A)**). Alternatively, the promotor, specific for the attached polymerase, may be linked to two separate sequences, namely the polymerase gene and the target gene. See **FIG. 3(B)**. In this instance, the amount of polymerase imitating at the promotor site will increase as the polymerase gene is transcribed and translated. Finally, the coding sequence transcribed by the T_7 promotor (or any specific first promotor) may produce any RNA polymerase (including T_7 polymerase or polymerase III or others), and this polymerase may transcribe off of another or second promotor (or off of a different strength T_7 or other first promotor) to produce the transcript of the target sequence. (See **FIG. 3(C)**).

[0095] Referring to the constructs or cassettes shown in **FIG. 4(A-C)**, these can be derived by using standard recombinant DNA techniques. The appropriate piece of DNA can be isolated and covalently attached to the RNA polymerase under conditions whereby the RNA polymerase functions after being covalently attached to a solid matrix (Cook, P. R and Grove, F. Nuc. Acids Res, 20:3591-3598. (1992)). Methods of modifying the ends of DNA molecules for attachment of chemical moieties are well known (see for example, U.S. patent application Ser. No. 08/032,769, supra). The transcribed product can act per se as sense or antisense RNA or it can be translated into protein. The enzyme and/or nucleic acid constructs could be modified to facilitate transport and/or achieve resistance to degrading enzymes (U.S. Pat. No. 5,241,060, supra).

[0096] (b) In vivo Amplification of Transcription

[0097] Constructs can be made that are dependent upon transcription or replication using a host polymerase. When such a construct contains a double promotor, the second promotor can be different than the first promotor or it can be a stronger or weaker version of the first promotor. Vectors can be chosen such that the constructs can either integrate into the chromosome, replicate autosomally or be replication deficient and function only for transient expression. They can function in the nucleus or the cytoplasm if the target cell is eukaryotic. The figure below depicts various constructs or cassettes and is not limiting as to the possible variations contemplated by the present invention.

[0098] Referring to **FIG. 4(A)**, the nucleic acid construct or cassette depicted in this figure contains a promotor that codes for the production of a polymerase that is not endogeneous to the target cell. For example, an SV40 or RNA polymerase III promotor that codes for a T_7 RNA polymerase. Transcription and translation of these transcripts by cellular machinery results in the production of active T_7 RNA polymerase which will utilize the T_7 promotor to transcribe the target sequence (Fuerst, T. R. et al., Proc Nat Acad Sci USA 83:8122 (1986)) have shown high levels of transient expression using a dual construct system with the T_7 RNA polymerase gene on one construct and the target gene behind the T_7 promotor on the other construct. The simplest iteration of this construct is that the genes coding for the polymerase code for a polymerase that exists within the cell and therefore is not recognized by the host organism as a foreign protein and does not induce an immune response.

[0099] In **FIG. 4(B)**, an additional autocatalytic cycle has been added whereby the extent of transcription of the target gene is enhanced by the production of T_7 RNA polymerase throughout the transient expression cycle.

[0100] In **FIG. 4(C)**, the construct or cassette is similar to **FIG. 4(B)** with the additional element that there is a down regulation of the autocatalytic cascade by competition by a high efficiency promotor with a low efficiency transcriptional promotor.

[0101] Three Constructs with Promotors for Endogenous RNA Polymerase

[0102] As described in the summary, the present invention further provides a construct comprising a host promoter located on the construct such that the host transcribes a sequence in the construct coding for a different RNA polymerase which after translation is capable of recognizing its cognate promoter and transcribing from a DNA sequence of interest in the construct with the cognate promoter oriented such that it does not promote transcription from the construct of the different RNA polymerase. In one feature of this construct, the host promotor comprises a prokaryotic promoter, e.g., RNA polymerase, or eukaryotic promoter, e.g., Pol I, Pol II, Pol III, or combinations thereof, such prokaryotic or eukaryotic promoter being located upstream from the host promoter. The second RNA polymerase may be selected from various members, including T7, T3 and SP6, or combinations thereof. The DNA sequence of interest may comprise sense or antisense, or both, or it may comprise DNA or RNA, or still yet, it may encode a protein. The construct may further comprise at least one chemically modified nucleoside.

[0103] Additionally, promotors that will be read by polymerases within the target cell can be linked to the production of additional polymerase specific for that promotor or other promotors. The polymerases can be for example, T7 poly-

merase, RNA polymerase III, or any other polymerase. A second promotor keyed sequence can be in the construct such that a second polynucleotide can be synthesized from the construct. It can code for the production of antisense or sense RNA or DNA molecules. These constructs or cassettes can be created using standard recombinant DNA techniques.

[0104] The property and structure of all nucleic acid constructs provided in accordance with this invention is applicable to each other in combination, in toto or in part. That is to say, in the conjugate comprising a protein and a nucleic acid construct, the construct could include, for example, chemical modification and bubble structure or single-stranded regions for primer binding sites or RNA initiation sites. Other variations would be recognized by those skilled in the art in light of the detailed description of this invention.

[0105] The examples which follow are set forth to illustrate various aspects of the present invention but are not intended to limit in any way the scope as more particularly set forth in the claims below.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

EXAMPLES

Example 1

[0106] Primers

[0107] A set of twenty single stranded oligonucleotide primers, fifteen nucleotides long, were chemically synthesized.

[0108] The first set of 10 primers was complementary to one strand of M13mp18 replicative form (RF) starting at base 650 and extending to base 341. An interval of 15 nucleotides separated successive primers. The second set of 10 primers contained sequences identical to the single-stranded M13mp18 phage genome starting at base 351 and extending to base 635, again with 15 nucleotide gaps separating successive primers. There is a complementarity of 5 bases between opposing primers, but at an ionic concentration of 0.08M NaCl and 45° C. these primers will not hybridize to each other. The sequences of the primers are shown in FIG. 6.

transform the circular RF molecule into a linear DNA molecule. The TaqI enzyme digests M13mp18 RF yielding 12 fragments. The sequence to be amplified (nucleotides 351 to 650) was flanked in the BamH1/EcoR1 digested RF by two regions, 1,371 bases and 5,601 bases, and TaqI-digested M13mp18 RF was flanked by regions of 15 and 477 nucleotides (see FIG. 7).

[0112] In amplification experiments, the restriction digests were used without any further purification. For amplification, a control of irrelevant DNA (calf thymus) was employed.

[0113] The precursors were added in 50 μl aliquots. One 10 μl aliquot of the precursors was mixed with 90 μl H_2O and loaded on a glass fiber filter, dried and counted. The counts were multiplied by 5 and divided by 160 (amoles in the incubation mix). Specific activity is the cpm/amoles of nucleotides.

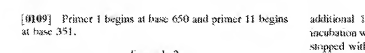
[0114] Amplification is measured as follows. The total counts are determined and this number is divided by the specific activity of the precursors to determine the number of nanomoles of incorporation. The target (in n grams) is divided by 330 (average molecular weight of nucleotide) to determine the nanomoles of input target phosphate. The amplification is then calculated by dividing the nanomoles of product by the nanomoles of input target.

Example 3

[0115] The Effect of Primer Concentration on the Amplification of Target DNA.

[0116] An incubation mixture of 130 μl contained the following reaction components: 40 mM sodium phosphate, pH 7.5, 400 μM each of the four deoxynucleotide triphosphates, 5 mM dithiothreitol, 40 ng of TaqI-digested M13mp18 RF (containing 3.5 ng of the TaqI fragment to be amplified), and all 20 primers (at 0.04 OD/ml, 0.4 OD/ml or 0.8 OD/ml) and 15 units of Klenow fragment of DNA polymerase. The mixture was left at room temperature for 20 minutes in order to allow the enzyme to cover all of the initiation sites on the template. The polymerization was then initiated by the addition of Mg^{++}, 7 mM final concentration, and the tubes were placed in a 45° C. bath. After 1 hour an

ARRANGEMENT OF OLIGONUCLEOTIDE PRIMERS IN AMPLIFICATION REACTION



[0109] Primer 1 begins at base 650 and primer 11 begins at base 351.

Example 2

[0110] Amplification Target

[0111] The target of amplification was the M13mp18 RF. This was digested with either TaqI or a combination of BamH1 and EcoR1. EcoR1 and BamH1 cut at sites close to each other and digestion with either enzyme alone would

additional 15 units of the enzyme were added, and the incubation was continued for another hour. The reaction was stopped with 100 nmoles of EDTA, 100 μg sonicated calf thymus DNA were added, and the nucleic acids were precipitated with 1.0 ml of cold 10% TCA for 60 minutes at 0° C. The mixture was filtered through a glass fiber filter, the filter was washed 3 times with cold 5% TCA, then twice with ethanol, dried and counted in a Beckman liquid scintillation counter.

[0117] The specific activity of the nucleotide precursors was 9,687 cpm/nmole. The tagged Taq1 DNA fragment contained 0.0106 nmoles of nucleotides.

Primer Concentration	Incorporation (cpm)	Incorporation (nmoles nucleotide)	Amplification
0.04 OD/ml	32,482	3.35	3.1b
0.4 OD/ml	366,260	37.8	35.6
0.8 OD/ml	512,260	52.81	49.8

Example 4

[0118] The Random Priming Activity of the Primers on Calf Thymus DNA.

[0119] To test for the effect of the primers on the background, an assay was performed, as described in the preceding example (Example 3 above), in which background was determined with and without primers as well as with and without melting of the calf thymus DNA.

[0120] The amplification conditions were the same as in Example 1 except that only 5 ug (15.0 nmoles) calf thymus DNA were used as a target. The DNA employed was double stranded or heated at 100° C. for 10 minutes in the presence or absence of primers (0.4 OD/ml each) before being chilled on ice.

Double Stranded DNA	Melted DNA	Primers	Incorporation cpm	Incorporation nmoles	Amplification
-			239,160	24.68	1.64
-		+	276,340	28.54	1.90
	+	+	556,360	57.45	3.83
	+		26,432	2.73	0.19

[0121] This experiment suggests that the random priming activity of the primers is not substantial, that incorporation on double stranded DNA is due to the nicks on the DNA molecules, and that melting abolishes to a large extent the priming positions on the irrelevant DNA.

Example 5

[0122] Amplification of the M13 Fragment in the Presence of a Large Excess (1,500-Fold) of Irrelevant DNA

[0123] The amplification conditions were the same as in Example 1. Primers (0.4 OD/ml), 5 ng calf thymus DNA and 40 ng M13mp18 DNA containing 3.5 ng of fragment were employed in this example.

Calf Thymus DNA	M13mp18 DNA	Incorporation cpm	Incorporation nmoles	Amplification
-		575,940	59.4	3.6ox
+	+	338,920	35.0	3,300x
+	+	715,840	73.8	

[0124] The experimental results above show that the target can be amplified in the presence of large amounts of irrelevant DNA. The net amplification was 1,343 even though in this case the target DNA inhibits the amplification of the irrelevant DNA by competing for initiation points. It is possible that the amplification was even larger.

[0125] These experimental results were also analyzed by running the samples on a 2% agarose gel. In FIG. 8 it can be seen that the calf thymus template (lane 3) only gives high molecular weight DNA bands composed of a mixture of input DNA as well as DNA synthesized by random priming (as seen in the incorporation figures in the Table above given for this example). On the other hand, it can be seen that the mp18 template (lane 2) gives a distinct pattern of lower molecular weight bands, and in lane 1, similar bands are observed when the mp18 template was mixed with 1500 times as much calf thymus DNA demonstrating that the foreign DNA did not significantly affect the amplification of DNA from the mp18 template.

Example 6

[0126] Amplification of Different Restriction Digests

[0127] The incubation conditions were the same as in Example 4. Forty nanograms of total M13mp18 DNA were used in each experiment with 0.4 OD/ml primers. In one case, the M13mp18 DNA was cut in only one position (using EcoR1) leaving the fragment to be amplified flanked by two large pieces. In the other case where the RF was treated with Taq1, the fragment was contained in one 639 base pair fragment. The specific activity of the precursors was 8,385 cpm/nmole.

	Incorporation cpm	Incorporation nmoles
Large Fragment	393,680	46.95
Small Fragment	262,808	31.34

[0128] These experimental results show that the enzyme does not extend polymerization very far from the region where the primers hybridize, otherwise a much larger incorporation using the large piece would have been otherwise expected because the elongation of the primers by the enzyme can extend in both directions.

Example 7

[0129] A Comparison of Synchronized and Unsynchronized Reactions

[0130] In all of the preceding experiments, the enzyme was preincubated with the target-primer mixture to allow binding of the enzyme at the 3' end of the hybridized primers on the target, followed by the addition of magnesium to initiate polymerization. The conditions were the same as in Example 1.

[0131] To assay the effect of this synchronization on the extent of the reaction, the incorporation in a synchronized reaction was compared to an unsynchronized reaction initiated by adding magnesium to the complete reaction mix before enzyme addition. The reaction conditions are described in Example 3. The specific activity was 9687 cpm/nmole

	Incorporation cpm	Incorporation cpm/hr	Amplification
Synchronized	405,000	51.3	4938
Unsynchronized	410,500	47.6	4952

[0132] The results above demonstrate that synchronization of the reaction is not essential for the amplification reaction.

Example 8

[0133] The Effect of Variations of the Reaction Conditions on the Product Produced by the Procedure of Example 3

[0134] A reaction was performed according the the reaction conditions of Example 3 in which twenty primers were added to the reaction mixture as well as the Taq I fragments (40 nanograms, i.e., 3.5 nanograms of insert that will hybridize with the primers) described in Example 3 with the exception that the buffer was altered. In the first lane of the gel shown in FIG. 9, the reaction was performed without target DNA added. In lane 2 the reaction was performed in a phosphate buffer (0.04 M, pH 7.5). Lane 3 contains the molecular weight buffers of Msp I digestion of pBR322 DNA. In the fourth lane the reaction was performed in which the phosphate buffer was substituted by MOPS buffer at 0.1 M and pH 7.5 (measured 25° C.). It can be seen that the reaction in the phosphate buffer produced an agglomeration of DNAs that when dissociated by heat or other double helix disrupting agents lead to an number of products of a size smaller than the agglomeration structures. The size distribution of the products in the MOPS-buffered reaction corresponds to the distances between certain of the oligo primer binding sites. The smallest is approximately 75 nucleotide pairs in size which is approximately the distance between the closest specific oligo primer binding sites.

Example 9

[0135] Effect of Various Buffers on the Amplification Reaction.

[0136] The buffer used for the amplification reaction can have significant effects upon the degree of amplification. In the following example, phosphate buffer (which was employed in Example 7) was compared with the following zwitterion buffers:

[0137] 4-morpholinoethyl sulfonate (MES),

[0138] 4-morpholinopropionyl sulfonate (MOPS),

[0139] N-dimethylaminobutyric acid (DMAB), and

[0140] N-dimethylglycene (DMG).

[0141] Trizma base was used to adjust MES or MOPS to pH 7.5, DMAB to pH 7.8, and DMG to pH 8.6. In the previous experiments, 4.0 ng of mp18 (containing 3.5 ng of the target fragment) was used as a template. In this experiment, the amount of template was reduced ten-fold compared to those experiments (4 ng of mp18; 350 pg of target fragment). Other changes in the experimental procedure was the omission of DTT and the use of a single addition of 10 units of Klenow polymerase. Mg^{++} and dNTP concentrations (7.5 mM and 400 μM each dNTP) were as described previously.

[0142] As before, reactions were preincubated at room temperature for 30 minutes prior to the addition of the Mg^{++}. After addition of Mg^{++}, reactions were immediately transferred to a 45° C. water bath and incubated for 4 hours. The reaction was stopped by the addition of 5 μl of 500 mM EDTA to give a final concentration of approximately 20 mM.

[0143] For evaluation of the extent of polymerization, an aliquot of 40 μl (out of a 120 μl incubation mix) was mixed with 50 μg of sonicated calf thymus DNA and precipitated on ice with 1 ml of 10% TCA. After one hour, the precipitate was collected on glass fiber filters, washed 3 times with 5% of cold TCA, 2 times with 95% ETOH, dried and counted in a liquid scintillation counter. The input was measured by the addition of radioactive precursor onto a filter without precipitation with TCA and counted as before. The results are given in the table below. As controls, the reactions were also carried out without the addition of any target template.

Buffer	Incorporation From Template (in cpm)	No Template Control (in cpm)	Template-Specific Incorporation (in cpm)	Net Synthesis (nanomoles)	Amplification Factor
Phosphate	4,005	2,020	1,360	0.255	240
MES	299,367	212,778	86,589	16.03	15,123
MOPS	284,560	49,521	114,979	23.28	20,975
DMAB	307,239	5,850	211,388	39.13	36,915
DMG	182,685	32,032	150,653	27.89	26,311

[0144] Compared to the no template control, the highest efficiency of amplification was obtained with the DMAB buffer. The results of this experiment demonstrated that an amplification of the target region approaching 37,000 fold could be obtained. It should be noted that another buffer, MES, gave higher incorporation, but the no template control demonstrated that there was non-specific polymerization leading to a net amplification of only 20,000 fold. The next best buffer system was DMG where net amplification was over 20,000 fold, followed by MOPS with 20,000 fold amplification.

[0145] The results of this experiment were also analyzed qualitatively by ethanol precipitating the remaining 80 μl of the reaction mixtures, resuspending them in 80 μl of TE

buffer and running 10 μl aliquots on 2% agarose gels. These results are shown in **FIG. 10** and agree with the results shown in the table above.

Example 10

[0146] Incorporation of radioactive precursors measures total synthesis of DNA including both specific as well as template-independent DNA synthesis. Oligos No. 1,3,5,7,9, 12,14,16,18 and 20 from Example 1 were employed in a series of amplification reactions. This limited number was chosen such that there would be a region within the amplicon that does not correspond to any of the primers, allowing the use of a 30 base probe (bases 469 to 498) labeled with biotin that corresponds to this open region.

[0147] The experimental design was to use DMAB and DMG buffers. Example 9 had previously shown little or no template-independent synthesis with DMAB and substantial template-independent synthesis with DMG. Reactions with and without Taq digested mp18 were carried out. The reaction mixtures were precipitated with ethanol, resuspended in TE buffer and aliquots were electrophoresed through a 2% agarose gel. A southern blot was made from this gel and probed with 200 ng/ml labeled oligo in 31% formamide/2xSSC at 25° C. for 2 hours and washed 3x with 0.1xSSC/0.1% Triton X-100 for 5 minutes each at 37° C. Membranes were developed using an alkaline phosphate detection system obtained from Enzo Biochem, Inc.

[0148] As seen in **FIG. 11**, this set of experiments demonstrates that the product of the amplification is strongly dependent upon the specific buffer used in the reaction. The best results were obtained with DMAB buffer where essentially no incorporation (data not shown) or hybridization (**FIG. 11**, lane 1) with the reaction mixture from the no template control sample. The template dependent synthesis with DMAB (**FIG. 11**, lane 2) produced DNA that hybridized with the labeled probe.

[0149] The nature and origin of the non-template derived synthesis achieved with DMG buffer (**FIG. 11**, lane 3) is still under current study

Example 11

[0150] Determination of the Nature of the Ends of the Amplified Product

[0151] If the product strands act as the template for polymerization of nucleic acid then the products should have blunt ends. One method of assaying for the presence of blunt ends is based on the notion that these molecules will undergo blunt end ligation. Molecules with 'ragged' ends (single stranded tails on the 3' or 5' end) will not participate in the ligation reaction.

[0152] Because the amplified product is initiated using chemically synthesized primer molecules, the 5' ends will not under phosphorylation. The first step of this proof will be to phosphorylate the 5' ends of both single stranded and double stranded molecules. These 5' phosphorylated molecules will then be ligated using the T4 DNA ligase. The unamplified DNA will act as the negative control and a PCR-generated amplicon will act as the positive control.

[0153] As can be seen in the gel reported in **FIG. 12**, T4 ligase treatment increases the molecular weight of the amplified product selectively. This is most apparent in lane 4 of **FIG. 12**, where there is an appreciable increase in size observed as a result of the completed ligation reaction.

[0154] The positive control with the PCR-generated amplicon (primed by oligos initiating at nucleotide 381 and from nucleotide 645, of the template which predicts an amplicon of 264 nucleotides) also show a shift in position after ligation (lane 7). Because there was not much DNA included in this reaction, the appearance of a spectrum of multimers of the amplicon was not observed, but the loss of material from the position of the unligated material (lanes 5 and 6) was noted. Some material left at the position of the unreated amplicon in lane 7 was also noted. It is possible that this material does not participate in the ligation reaction because of the addition of A to the 3' end of the amplicon which is a property of the Taq polymerase.

Example 12

[0155] Amplification from Non-Denatured Template

[0156] To explain the high level of amplification in this system, it was assumed that some of the primers might be able to initiate DNA synthesis by inverting the ends of double-stranded DNA products synthesized during amplification. This "breathing" was demonstrated in the following experiment. The template was a blunt-ended double-stranded DNA molecule obtained from Dr. Christine I. Brakel, the blunt ends extending from bases 371 to 645 in the mp18 DNA sequence. These ends exactly match primers Nos. 1 and 12 (described in Example 1). In this experiment, no radioactive precursors were used. Analysis was performed by gel electrophoresis through 2% agarose. Reagent conditions were the same as Example 10 where DMG was used as the buffer, but only 2 primers, No. 1 and No. 2 were used and no denaturation of the starting template was performed. In **FIG. 13**, for comparison purposes, the same amount of DNA was used as the input on the gel (lane 1). In lane 2, no template was added. In lane 3, the complete reaction mix is shown. In lane 4, 12 times as much DNA as the template input in either lanes 1 or 3 has been included as a size marker. In both lanes 2 and 3, some non-specific synthesis can be seen, but the specific copying of the template can clearly be distinguished in lane 3. Therefore, as lane 3 indicates, newly synthesized DNA of the same size as the input was formed using non-denatured double-stranded DNA, proving that the double-stranded blunt ends can serve as initiation points for replication.

Example 13

[0157] Amplication from RNA Template

[0158] Although it has been demonstrated by the present invention that DNA can be amplified, it would be useful, however, to also be able to employ RNA as a potential template. Accordingly, the double-stranded DNA molecule used in Example 12 was ligated into the Sma I site of a vector pHH31 (obtained from International Biotechnology Corp) that contains a promotor for T7 RNA polymerase. Using standard methodologies, an RNA transcript was synthesized encompassing the same sequences used in example 12. This transcript was amplified using standard conditions with all 20 primers in DMG buffer. Taq digested mp18 DNA was used as a control. As seen in **FIG. 14** there was extensive synthesis. There are characteristic bands that

appear in lane 4, the reaction with the RNA template, as well as in lane 2, the reaction with the DNA template that do not appear in the template independent synthesis seen in lanes 1 and 5.

[0159] This demonstrates that the system is capable of utilizing the RNA transcript as a template without the introduction of any other enzyme besides the Klenow, thus proving that the Klenow enzyme can be used as a reverse transcriptase as indicated in the disclosure. This result was studied further by making a Southern blot of the gel seen in FIG. 14 and probing with nick-translated biotinylated mp18 DNA using a nick translation kit obtained from Enzo Biochem, Inc. As seen in FIG. 15, there was little or no hybridization of the probe to the reaction product of the template independent reactions (lanes 1 and 5) whereas extensive hybridization was observed with the RNA derived reaction (lane 4) as well as the DNA template derived reaction (lane 2), as expected.

Example 14

[0160] Strand Displacement Using Ethidium-Labeled Oligonucleotides

[0161] Three oligonucleotides with the sequence listed in FIG. 16 were prepared and labeled F1, F1C and F3. The unlabeled complement of F1C was hybridized to unlabeled F1. The ratio of F1C: F1 for the hybridization was 1:2. (F1C at a concentration of 0.13 O.D/ml and F1 at a concentration of 0.26 O.D./mL) Hybridization was performed in 1xSSC for two hours at 45° C.

[0162] Aliquots of the hybrid were mixed with different amounts of ethidium-labeled F1 (F1E) in 1xSSC and incubated for 18 hours either at 43° C. or at 37° C. The ratios of F1E oligo to the unlabeled oligo F1C was 1:1, 2:1, 3:1 and 4:1. (The 1:1 reaction contained 0.0325 O.D of the F1E, 0.065 O.D. of F1 and 0.0325 O.D. of F1C.) At the end of the incubation period, 50 μl of each mixture was incubated with 50 μl of diazonium mixture for 5 minutes at room temperature. To prepare the diazonium mixture, 10 μl of the diazonium stock solution, (50 mM in 1M HCl), was added to 100 μl of cold dilution buffer, (1xSSC and 0.2 M $KHCO_3$, prepared fresh). The diazonium stock solution is stored at −20° C.

[0163] Under these conditions the diazonium will destroy the fluorescence associated with the ethidium in single stranded oligonucleotides. See, e.g., European Patent Application Publication No. 0 492 570 A1, published on Jul. 1, 1992, based on priority document, U.S. patent application Ser. No. 07/633,750, filed on Dec. 24, 1990, the contents of which are incorporated by reference. But the diazonium will not destroy the fluorescence associated with the ethidium that has intercalated into the double stranded DNA. The survival of the ethidium, under these reaction conditions, is a measure of the extent of formation of a double helix by the ethidium-labeled oligonucleotides, thus indicating displacement of the non-ethidium containing strand by that of the ethidium labeled. This property of the ethidium labeled oligonucleotides by primers can be usefully employed to facilitate initiation of polymerization on double stranded templates. As seen in the figure in FIG. 17, the ethidium-labeled oligo displaces the non-ethidium-labeled oligo better at 43° C. than at 37° C.

Example 15

[0164] T7 Promoter Oligonucleotide 50 Mer Labeled with Ethidium

[0165] An oligonucleotide 50-mer including the T7 promoter region of IBI 31 plasmid constructs (plasmid sequences derived from manufacturer, International Biotechnology, Inc.) was synthesized. Its sequence is as follows:

5'-TAC T*AA T*TC GCT* CT*A T*AG T*T--AA TCA TGA

AT---T AAT* TAT* GCT* GAG T*CA T*AT* C-5',

[0166] where T* represents allylamine dU, and therefore ethidium modification and the 10 base sequence set off by dashes (----AA TCA TGA AT----) was introduced to provide a restriction enzyme site.

Example 16

[0167] Use of the Oligonucleotide 50-Mer to Regulate RNA Synthesis In Vitro

[0168] This nucleotide is complementary to the ATG strand of the lac z gene of IBI 31, and also contains a 10-base sequence for use in restriction enzyme digestion. The oligonucleotide 50-mer also contains sequences overlapping the T7 promoter in the IBI 31 plasmid constructs. Thus, it might be expected to interfere with in vitro transcription by T7 RNA polymerase even though the sequences in this oligo are entirely upstream of the start of transcription by T7 RNA polymerase. Because the plasmid constructs contain opposing T7 and T3 promotors, this also means that the oligo 50-mer is identical in sequence to the RNA that is made by the T3 RNA polymerase in vitro.

[0169] The effect of this oligonucleotide on in vitro transcription by T7 and T3 polymerases from an IBI 31 plasmid construct (pIBI 31-BH5-2) and from a BlueScript II plasmid construct (pBSII/HCV) was studied. See FIG. 18 which contains the same target sequences, but in a "split" arrangement. The polylinker sequences of these plasmids are given in FIG. 18. Comparing the effect of the oligo on these two different target template serves to partially control for the possible non-specific inhibitory effects of ethidium groups on the RNA polymerases because the oligonucleotide would be expected to inhibit transcription from any template containing an appropriate promotor regardless of the "split" if the effect were due to the oligo's interaction with the polymerase rather than with the template.

[0170] At a concentration of 60-fold excess of oligonucleotide (0.6 pM final) over plasmid with either the allylamine labelled oligonucleotide of the ethidium labelled oligonucleotide in a transcription reaction mixture, the following results were obtained:

Plasmid Transcribed	Polymerase Used	Oligo Used	nanomoles Incorporated	% of control
pIBI 31-BH5-2	T3	None	246	100
pIBI 31-BH5-2	T3	Allylamine labeled	233	95
pIBI 31-BH5-2	T3	Ethidium labeled	93	37
pIBI 31-BH5-2	T7	None	208	100

-continued

Plasmid Transcribed	Polymerase Used	Oligo Used	nanomoles Incorporated	% of control
pBH 31-BH5-2	T7	Allylamine labeled	198	95
pBH 31-BH 5-2	T7	Ethidium labeled	3	1.4
pBSII/HCV	T3	None	132	100
pBSII/HCV	T3	Allylamine labeled	158	>100
pBSII/HCV	T3	Ethidium labeled	185	>100
pBSII/HCV	T7	None	125	100
pBSII/HCV	T7	Allylamine labeled	134	>100
pBSII/HCV	T7	Ethidium labeled	62	50

[0171] These results indicate that the ethidium-modified oligo sequence is capable of specifically inhibiting transcription by the T7 polymerase from the T7 promotor region provided that the promoter region is not interrupted by the multiple cloning region and inserted DNA. Thus, the effect is dependent on the template DNA and is not merely the result of inhibition of the T7 polymerase by the ethidium groups.

[0172] Many obvious variations will be suggested to those of ordinary skill in the art in light of the above detailed description of the invention. All such variations are fully embraced by the scope and spirit of the present invention as set forth in the claims which follow.

SEQUENCE LISTING

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<210> SEQ ID NO 1
<211> LENGTH: 7249
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      M13mp18 nucleotide sequence

<400> SEQUENCE: 1

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atagctaaac aggttattga ccatttgcga aatgtatcta atggtcaaac taaatctact 120

cgttcgcaga attgggaatc aactgttaca tggaatgaaa cttccagaca ccgtacttta 180

gttgcatatt taaaacatgt tgagctacag caccagattc agcaattaag ctctaagcca 240

tccgcaaaaa tgacctctta tcaaaaggag caattaaagg tactctctaa tcctgacctg 300

ttggagtttg cttccggtct ggttcgcttt gaagctcgaa ttaaaacgcg atatttgaag 360

tctttcgggc ttcctcttaa tctttttgat gcaatccgct ttgcttctga ctataatagt 420

cagggtaaag acctgatttt tgatttatgg tcattctcgt tttctgaact gtttaaagca 480

tttgaggggg attcaatgaa tatttatgac gattccgcag tattggacgc tatccagtct 540

aaacatttta ctattacccc ctctggcaaa acttcttttg caaaagcctc tcgctatttt 600

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atgaatcttt ctacctgtaa taatgttgtt ccgttagttc gttttattaa cgtagatttt 780

tcttcccaac gtcctgactg gtataatgag ccagttctta aaatcgcata aggtaattca 840

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1. (canceled)

2-90. (canceled)

91. An in vitro process for producing more than one copy of nucleic acids of interest, said process comprising the steps of:

(a) providing a nucleic acid sample containing said nucleic acids of interest;

(b) contacting said sample with a mixture comprising:

(i) nucleic acid precursors;

(ii) one or more specific polynucleotide primers comprising at least one ribonucleic acid segment, each of which primer comprises a sequence complementary to a distinct sequence of said nucleic acids of interest;

(iii) an effective amount of a nucleic acid producing catalyst; and

(iv) RNase H; and

(c) carrying out nucleic acid synthesis, thereby generating multiple copies of said nucleic acids of interest.

92. The process of claim 91, wherein said primers (ii) comprise modified nucleotides, unmodified nucleotides or a combination thereof.

93. The process of claim 91, wherein said primers (ii) comprise sequences noncomplementary to said distinct sequence of said nucleic acids of interest.

94. The process of claim 93, wherein said primers (ii) comprise from about 1 to about 200 noncomplementary nucleotides or nucleotide analogs.

95. The process of claim 91, wherein said primers (ii) further comprise deoxyribonucleotides.

96. The process of claim 91, wherein said nucleic acid producing catalyst (iii) comprises DNA polymerase, RNA polymerase, reverse transcriptase or a combination thereof.

97. The process of claim 96, wherein said DNA polymerase comprises *E. coli* DNA polymerase I, Klenow polymerase, polymerases derived from thermophilic bacteria or a combination thereof.

98. The process of claim 97, wherein said polymerases derived from thermophilic bacteria comprise Taq DNA polymerase.

99. The process of claim 91, wherein said mixture recited in step (b) comprises nucleic acid precursors, one or more specific labeled polynucleotide primers or a combination of both.

100. The process of claim 91, wherein said primers comprise a 3'-hydroxyl group or an isosteric configuration of heteroatoms.

101. The process of claim 100, wherein said heteroatoms comprise nitrogen or sulfur.

102. An in vitro process for producing more than one copy of nucleic acids of interest, said process comprising the steps of:

(a) providing a nucleic acid sample containing or suspected of containing said nucleic acids of interest;

(b) contacting said sample with a mixture comprising:

(i) nucleic acid precursors;

(ii) one or more specific polynucleotide primers comprising at least one ribonucleic acid segment and at least one deoxyribonucleic acid segment, each of which primer comprises a sequence complementary to a distinct sequence of said nucleic acids of interest;

(iii) an effective amount of a nucleic acid producing catalyst; and

(iv) RNase H; and

(c) allowing nucleic acid synthesis to be carried out, thereby generating multiple copies of said nucleic acids of interest

103. The process of claim 102, wherein said primers comprise modified nucleotides, unmodified nucleotides or a combination of both.

104. The process of claim 102, wherein said primers comprise sequences noncomplementary to said distinct sequence of said nucleic acids of interest.

105. The process of claim 104, wherein said primers comprise from about 1 to about 200 noncomplementary nucleotides or nucleotide analogs.

106. The process of claim 102, wherein said nucleic acid producing catalyst (iii) comprises DNA polymerase, RNA polymerase, reverse transcriptase or a combination theeof.

107. The process of claim 106, wherein said DNA polymerase comprises *E. coli* DNA polymerase I, Klenow polymerase, polymerases derived from thermophilic bacteria, or a combination thereof.

108. The process of claim 107, wherein said polymerases derived from thermophilic bacteria comprise Taq DNA polymerase.

109 The process of claim 102, wherin said mixture recited in step (b) comprises nucleic acid precursors, one or more specific labeled polynucleotide primers, or a combination of both.

110. The process of claim 102, wherein said primers (ii) contain a 3'-hydroxyl group or an isosteric configuration of heteroatoms.

111. The process of claim 110, wherein said heteroatoms comprise nitrogen or sulfur.

112. An in vitro process for producing more than one copy of nucleic acids of interest, said process comprising the steps of:

(a) providing a nucleic acid sample containing said nucleic acids of interest;

(b) contacting said sample with a mixture comprising:

(i) nucleic acid precursors;

(ii) one or more specific polynucleotide primers comprising at least one ribonucleic acid segment, each of which primer comprises a sequence complementary to a distinct sequence of said nucleic acids of interest;

(iii) an effective amount of a nucleic acid producing catalyst; and

(iv) RNase H; and

(c) carrying out nucleic acid synthesis to produce a polynucleotide comprising an RNA/DNA hybrid, thereby generating a substrate for RNase H;

(d) digesting said substrate with RNase H to remove said ribonucleic acid segment and allow another primer binding event to occur, thereby producing multiple copies of said nucleic acids of interest.

113 The process of claim 112, wherein said primers (ii) comprise modified nucleotides, unmodified nucleotides or a combination thereof.

114 The process of claim 112, wherein said primers (ii) comprise sequences noncomplementary to said distinct sequence of said nucleic acids of interest.

115. The process of claim 114, wherein said primers (ii) comprise from about 1 to 200 noncomplementary nucleotides or nucleotide analogs.

116 The process of claim 112, wherein said primers (ii) further comprise deoxyribonucleotides.

117. The process of claim 112, wherein said nucleic acid producing catalysts (iii) comprise DNA polymerase, RNA polymerase, reverse transcriptase or a combination thereof.

118. The process of claim 117, wherein said DNA polymerase comprises *E. coli* DNA polymerase I, Klenow polymerase, polymerases derived from thermophilic bacteria or a combination thereof.

119 The process of claim 118, wherein said polymerases derived from thermophilic bacteria comprise Taq DNA polymerase.

120. The process of claim 112, wherein said mixture recited in step (b) comprises nucleic acid precursors, one or more specific labeled polynucleotide primers, or a combination of both.

121. The process of claim 112, wherein said primers (ii) contain a 3'-hydroxyl group or an isosteric configuration of heteroatoms.

122. The process of claim 121, wherein said heteroatoms comprise nitrogen or sulfur.

123. A process for multiply initiating polynucleotide or oligonucleotide synthesis comprising:

(a) providing nucleic acids of interest;

(b) contacting said sample with a mixture comprising:

(i) nucleic acid precursors;

(ii) one or more specific copolymer primers comprising at least one DNA segment and at least one RNA segment, each of which primer comprises a sequence complementary to a distinct sequence of said nucleic acid of interest;

(iii) an effective amount of a nucleic acid producing catalyst; and

(iv) RNase H; and

(c) producing at least one copy of said nucleic acid of interest by using said nucleic acid producing catalyst (iii) and said nucleic acids of interest as templates; and

d) removing said RNA segment from said template by digesting with RNase H to bind another primer and initiate synthesis, thereby multiply initiating polynucleotide or oligonucleotide synthesis.

124. The process of claim 123, wherein said primers comprise modified nucleotides, unmodified nucleotides or a combination thereof.

125. The process of claim 123, wherein said primers further comprise sequences that are noncomplementary to said distinct sequence of said nucleic acids of interest.

126. The process of claim 125, wherein said primers comprise from about 1 to 200 noncomplementary nucleotides or nucleotide analogs.

127. The process of claim 123, wherein the nucleic acid producing catalyst (iii) comprises DNA polymerase, RNA polymerase, reverse transcriptase or a combination thereof.

128. The process of claim 127, wherein said DNA polymerase comprises *E. coli* DNA polymerase I, Klenow polymerase, polymerases derived from thermophilic bacteria or a combination thereof.

129. The process of claim 128, wherein said polymerases derived from thermophilic bacteria comprise Taq DNA polymerase.

130. The process of claim 123, wherein said mixture recited in step (b) comprises nucleic acid precursors, one or more specific labeled polynucleotide primers or a combination of both.

131. The process of claim 123, wherein said primers contain a 3'-hydroxyl group or an isosteric configuration of heteroatoms.

132. The process of claim 131, wherein said heteroatoms comprise nitrogen or sulfur.

133. An in vitro process for producing more than one copy of RNA of interest, said process comprising the steps of:

(a) providing a nucleic acid sample containing said RNA of interest;

(b) contacting said sample containing with a mixture comprising:

(i) nucleic acid precursors;

(ii) one or more polynucleotide primers wherein said primers comprise (A) at least one ribonucleic acid segment and (B) a sequence complementary to a distinct sequence in said RNA of interest;

(iii) an effective amount of a nucleic acid producing catalyst; and

(iv) RNase H;

(c) producing at least one DNA copy from said RNA of interest;

(d) using said DNA copy as a template to produce a double-stranded copy comprising a second copy complementary to said DNA copy produced in step (c); and

(e) removing said ribonucleic acid segment of said primers with RNase H from said double-stranded copy produced in step (d) to regenerate a primer binding site, thereby allowing a new priming event to occur and producing more than one copy of said RNA of interest.

134. The process of claim 133, wherein said primers (ii) comprise modified nucleotides, unmodified nucleotides or a combination thereof.

135. The process of claim 133, wherein said primers (ii) further comprise sequences noncomplementary to said distinct sequence of said RNA of interest.

136. The process of claim 135, wherein said primers (ii) further comprise from about 1 to 200 noncomplementary nucleotides or nucleotide analogs.

137. The process of claim 133, wherein said primers (ii) further comprise deoxyribonucleotides.

138. The process of claim 133, wherein said nucleic acid producing catalysts (iii) comprise DNA polymerase, RNA polymerase, reverse transcriptase or a combination thereof.

139. The process of claim 138, wherein said DNA polymerase comprises *E. coli* DNA polymerase I, Klenow polymerase, polymerases derived from thermophilic bacteria, or a combination thereof.

140. The process of claim 139, wherein said polymerases derived from thermophilic bacteria comprise Taq DNA polymerase.

141. The process of claim 91, wherein said mixture recited in step (b) comprises nucleic acid precursors, one or more specific labeled polynucleotide primers or a combination of both.

142. The process of claim 141, wherein said primers comprise from about 1 to 200 noncomplementary nucleotides or nucleotide analogs.

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